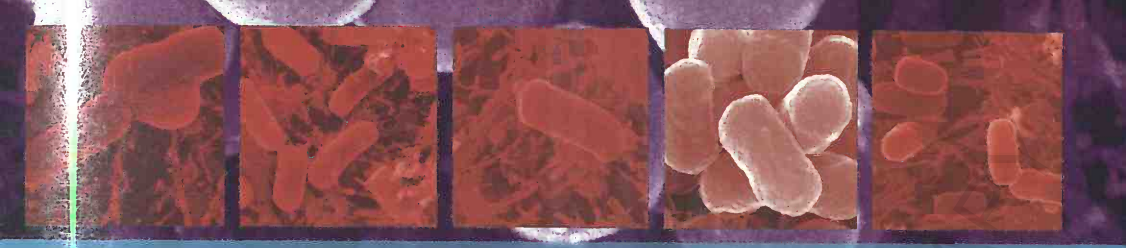


APPLIED MICROBIOLOGY

FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS

Marius Ştefan

III 314.169

Editura Universităţii „Alexandru Ioan Cuza" Iaşi

Marius Ștefan

APPLIED MICROBIOLOGY: FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS

BCU IASI / CENTRAL UNIVERSITY LIBRARY

The book was published in relation with Romania-France bilateral cooperation research project no. 780/30.06.2014 - *Applications of some biomolecules with antifungal properties in plant protection*

Scientific Reviewers:
Prof. dr. Mariana Carmen CHIFIRIUC
Associate prof. dr. Zenovia OLTEANU

Contributors:
Lucian Hriţcu, Marius Mihăşan

Coverage: Manuela Oboroceanu

ISBN: 978-606-714-091-0

This book is for education and research use only, not for sale or other commercial purposes.

© Editura Universităţii „Alexandru Ioan Cuza", Iaşi, 2014
700109 – Iaşi, str. Pinului, nr. 1A, tel./fax: (0232) 314947
http:// www.editura.uaic.ro e-mail: editura@uaic.ro

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

Marius Ștefan

APPLIED MICROBIOLOGY: FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS

207114

Preface by Veronica Lazăr

ALE

EDITURA UNIVERSITĂŢII „ALEXANDRU IOAN CUZA”
IAŞI – 2014

0 000010 32832 BCU IAȘI

BCU IAȘI / CENTRAL UNIVERSITY LIBRARY

Descrierea CIP a Bibliotecii Naţionale a României
ŞTEFAN, MARIUS

Applied microbiology: from plant growth promotion to new neuroprotective drugs / Marius Ştefan; contributors: associate prof., PhD, dr. habil. Lucian Hriţcu, associate lecturer, PhD, Marius Mihăşan; pref. de Veronica Lazăr. - Iaşi: Editura Universităţii „Al. I. Cuza", 2014

Bibliogr.

ISBN 978-606-714-091-0

I. Hriţcu, Lucian
II. Mihăşan, Marius
III. Lazăr, Veronica (pref.)

579

2 0 MAR. 2015

BCU "ST. CENTRAL UNIVERSITY LIBRARY

This book is dedicated to my family for the years of support, tolerance and patience

Special thanks to Marius - friend, teacher, collegue. I am gratefull to Lucian - reliable collaborator and to all my collegues. My sincere gratitude goes to Prof. Constantin Toma - remarkable man, outstanding academic example.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Contents

Preface (*Veronica Lazăr)* 9

Chapter 1. PLANT-MICROBIAL INTERACTIONS IN THE RHIZOSPHERE – STRATEGIES FOR PLANT GROWTH-PROMOTION 15

Introduction 16

1.1. SCREENING FOR PLANT GROWTH PROMOTING RHIZOBACTERIA 33

1.1.1. Characterization of bacterial strains isolated from soybean rhizosphere 33

1.1.2. Characterization of bacterial strains isolated from runner bean rhizosphere 41

1.2. RHIZOBACTERIA USED AS BIOFERTILIZERS 51

1.2.1. Plant growth promoting rhizobacteria can inhibit the germination of soybean seeds 51

1.2.2. Soybean inoculation with *Bacillus pumilus* Rs3 promotes plant growth and increases seed protein yield 56

1.2.3. Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean 62

Chapter 2. ANTIMICROBIAL APPLICATIONS OF DIFFERENT NATURAL AND ARTIFICIAL COMPOUNDS 79

Introduction 80

2.1. COMPOSITION AND ANTIBACTERIAL ACTIVITY OF BASIL ESSENTIAL OILS 80

2.2. ANTIBACTERIAL ACTIVITIES OF SOME METHANOLIC PLANT EXTRACTS AGAINST MULTIDRUG-RESISTANT BACTERIA 90

BCU "Ion Ghica" / CENTRAL UNIVERSITY LIBRARY

2.2.1. Antibacterial activities of some vegetables extracts 91

2.2.2. Antimicrobial and antibiotic-potentiation activities of some edible plants extracts 103

2.3. ANTIBACTERIAL EFFECT OF NOBLE METAL NANOPARTICLES 117

2.3.1. Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media 118

2.3.2. Antimicrobial properties of chitosan capped gold nanoparticles ... 130

Chapter 3. NEW BIOTECHNOLOGICAL APPLICATIONS OF THE METABOLIC DERIVATIVES FROM *ARTHROBACTER NICOTINOVORANS* 139

Introduction 140

3.1. EVIDENCE OF A PLASMID-ENCODED OXIDATIVE XYLOSE CATABOLIC PATHWAY IN *ARTHROBACTER NICOTINOVORANS* pAO1 142

3.2. NICOTINE DERIVATIVES FROM *ARTHROBACTER NICOTINOVORANS* – NOVEL NEUROPROTECTIVE DRUGS? 156

3.2.1. *In-silico* identification of 6-hydroxy-L-nicotine as a novel neuroprotective drug 157

3.2.2. 6-hydroxy-L-nicotine from *Arthrobacter nicotinovorans* sustain spatial memory formation by decreasing brain oxidative stress in rats ... 161

CONCLUSIONS 167

REFERENCES 171

BCU IASI CENTRAL UNIVERSITY LIBRARY

Preface

One of the most applicative and large field of the Life Sciences is the General and Applied Microbiology field, which is covering under its umbrella a lot of applicative sciences, mainly the medical microbiology, microbial ecology and biotechnology; the last one, is based on the diversity and intensity of bacterial/microbial metabolism and includes traditional biotechnologies, very old and still useful at present time, but even modern biotechnologies based on the molecular biology techniques. The directions of development of modern biotechnology processes are determined, on the other hand, by the problems facing the world today: the impending exhaustion of fossil fuels, the insufficient food reserves considering the boom demographic problem, the planetary ecological imbalances due to the irrational exploitation of resources, the intense, chronic pollution with organic matter and xenobiotics too, which led to the phenomenon of global warming resulting in drastic climate changes, which are also influencing the microbial growth rate and fitness of environmental pathogens. If we consider also the global phenomenon of antibioresistance and resistance genes pool (clinical and environmental reservoirs) it is obvious the necessity of new antimicrobials. All these problems are very complex and require the application of ecological solutions to stop or even reduce these negative effects of irresponsible attitude of man by many ways, including some microbial biotechnologies, in order to solve these difficulties: the energy crisis by finding reliable alternatives, including some biofuels, getting large amounts of fodder and

B.C.U. „M. EMINESCU" IAŞI

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

alimentary proteins, new antimicrobials, bioremediation of chronically polluted soils and wastewater treatment, biodegradation of human activity wastage, readily biodegradable or refractory to microbial attack and reintroduction of biogenic elements in their natural circuit.

In the actual context, the research and educational book conceived as a monographic work "APPLIED MICROBIOLOGY: FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS" is an example of applied microbiology/ biotechnology, its main author and coauthors presenting their own research experience. This book of applied microbiology is structurated in three chapters dedicated to: I. Plant-microbial interactions in the rhizosphere – strategies for plant growth promotion; II. Antimicrobial applications of different natural and artificial compounds; III. New biotechnological applications of the metabolic derivatives from *Arthrobacter nicotinovorans*. This diversity of these apparently unrelated subjects, but having a common denominator, are reflecting the huge applicative aria of microbiology knowledge, of bacteria and other microorganisms with different metabolic particularities, used in different biotechnologies/nanobiotechnologies and their great potential to offer ecological solutions at different problems in agriculture, medicine and even for its own branch – clinical microbiology, such as:

1) The microbial population of the rhizosphere as the effect of the plant roots on the surrounding soil and many reports described the soil attached to the root system as a hot spot of microbial abundance and activity. The root system, which was traditionally thought to provide only anchorage and uptake of nutrients and water, is considered by the author

BCU MSI CENTRAL UNIVERSITY LIBRARY

such as a biochemical "factory" that mediates numerous underground interactions. From this point of view the rhizosphere is a very complex "ecosystem" in which the effects of the plant on soil microorganisms and the effects of the microorganisms on the plant are interacting and dependent of the the intra- and interspecies communication systems. Plant root exudates attract microbes and feed them and, in turn, the plants often benefit from the microbes, of their synergistic metabolic activities and even from their antagonistic effect against phytopathogens. Plant growth-promoting rhizobacteria are a group of bacteria that can actively colonize plant roots and may have direct or indirect effects on health, growth and development of plants; these rhizosphere bacteria, selected and characterized, can be used as: biofertilizers (bacteria whith biological nitrogen fixation and phosphorus solubilization properties, siderophore production), phytostimulators (microorganisms with the ability to produce phytohormones) and biopesticides or biocontrol agents (microorganisms that promote plant health by controlling the phytopathogens); remarcably, the authors of this book succeeded to select some bacterial strains able to combine all these desirable properties.

2) Despite the impressive scientific progress in vaccination and chemotherapy, infectious diseases remain a serious health issue. Under the selective pressure of therapeutical antibiotics, used excessively during the last decades, some preexistent bacterial species/strains harboring resistance genes (offering them protection against antibiotics naturally produced by themself or by other members of the microbial communities) were selected and disseminated, even developing other various

BCU IASI / CENTRAL UNIVERSITY LIBRARY

mechanism of resistance; by consequence, infectious diseases remain one of the leading causes of morbidity worldwide. Moreover, there are also a lot of biofilm associated infections, the biofilm embedded cells showing a different form of resistance, called now tolerance. Therefore the pharmaceutical companies and the researchers are in a continuous quest for new antibacterial agents for resistant/multiresistant strains, but also active agents, able to penetrate the biofilms and active on adherent cells. In this direction, some studies were focused on the investigation of the antibacterial effects of different natural (plant extracts) and artificial (noble metal nanoparticles) compounds. Concerning the plant extracts, apparently nothing new, they are used empiricaly since antiques period, but now they are investigated at molecular level, because their use have to be scientifically based, in specific amounts and for a specific target, comparable with the allopatic drugs; the experimental studies of the authors considering the synergistic activity with antibiotics and their possible failure due to the bacterial efflux pumps are interesting and probably this topic have to be continued.

3) The biotechnological applications of the actinobacterium species *Arthrobacter nicotinovorans,* named after its peculiar metabolic ability to metabolize the alkaloid nicotine, used as carbon and nitrogen source and of other identified metabolic products were delayed. Therefore, an important goal and challenge in the same time was to discover new biotechnological applications of some metabolic products of *Arthrobacter nicotinovorans* cells:

BCU IASI / CENTRAL UNIVERSITY LIBRARY

- firstly, the intermediate metabolites such as enzymes involved in the lignocellulose biodegradation are providing a promising alternative for replacing oil based chemicals and fuels due to its high abundance and renewable character. Since the D-xylose fraction of lignocellulose is rather significant, making up to 20% of its biomass, it is essential that this fraction be efficiently used to obtain cost-effective biochemical production. Although, theoretically, D-xylose can be readily exploited as a valuable resource for production of ethanol and various chemicals, in practice, such applications are scarce mainly due to the lack of suitable enzymes, stable and efficient; such enzymes could be produced by some species of soil bacteria occupying an ecological niche rich in lignocellulosic materials, such as *A. nicotinovorans* – an appropriate candidate for finding new degradation pathways and new xylose-degrading enzymes. The sugar-catabolic pathway is comprised of several genes, among which a putative cellulase, an ABC transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases; the three genes of this operon coding for the putative enzymes implicated in this pathway were cloned, purified and characterized by the authors of this research work;

- secondly, the metabolic use of nicotine (the main alkaloid produced by tobacco plant) by *A. nicotinovorans* is also based on the catabolic machinery encoded by pAO1 megaplasmid. Numerous studies focused primarily on the neuropharmacological effects of nicotine indicated an important impact of this alkaloid and his metabolites on brain functions. Thus it has become increasingly important to obtain more

BCU IASI / CENTRAL UNIVERSITY LIBRARY

information on the biological effects of nicotine metabolites on mammalian cells, in order to possibly manage neurological abnormalities in some neurodegenerative afflictions. Therefore, some experimental studies were designed by the authors in order to determine whether 6-hydroxy-L-nicotine, a nicotine metabolite derived from nicotine catabolism in *A. nicotinovorans*, affects spatial memory formation and antioxidant status, the results of these experiments showing that the spatial memory formation is sustained by decreasing brain oxidative stress.

All approached topics, but especially this last one are representing evidences for the previous mentioned idea: the huge range of applications of microbiology knowledge, of some selected microbial species/strains, used in high effective power biotechnologies for the human and environment benefits. Considering all these arguments, the present work has a great scientific and also an educational value.

Prof. dr. Veronica Lazăr
Faculty of Biology
The University of Bucharest

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Chapter 1.

PLANT-MICROBIAL INTERACTIONS IN THE RHIZOSPHERE – STRATEGIES FOR PLANT GROWTH-PROMOTION

Marius ȘTEFAN

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Introduction

Since the german agronomist Hiltner first defined in 1904 the rhizosphere as the effect of the roots of legumes on the surrounding soil, many reports presented the soil attached to the root system as a hot spot of microbial abundance and activity due to the presence of root exudates and rhizodeposits (Smalla, Sessitsch et al., 2006). The root system, which was traditionally thought to provide anchorage and uptake of nutrients and water, is in fact a chemical factory that mediates numerous underground interactions (Badri, Weir et al., 2009). From this point of view the rhizosphere is a very complex environment in which the effects of the plant on soil microorganisms and the effects of the microorganisms on the plant are interacting and interdependent (Mukerji, Manoharachary et al., 2006). Plant root exudates attract microbes and feed them and, in turn, the plants often benefits from the microbes.

Plant growth-promoting rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant roots and may have direct or indirect effects on health, growth and development of plants (Kuklinsky-Sobral, Araújo et al., 2004).

Rhizosphere colonization

Microbial attachment to and proliferation on roots is generally referred to as root colonization. Root colonization is an important factor in plant pathogenesis of soil-borne microorganisms as well as in

BCU IASI / CENTRAL UNIVERSITY LIBRARY

beneficial interactions used for microbiological control, biofertilisation, phytostimulation and phytoremediation (Thomas, Woeng et al., 2008).

The beneficial effect of PGPR on plant growth via an enhancement of the nutrient status can vary depending on where and how the PGPR colonizes the host plant. Since the 1980's, many studies have focused on the root colonization. PGPR may colonize the rhizosphere, the surface of the root, or even superficial intercellular spaces (McCully, 2001). Howie and co-workers hypothesized a two phase process (Howie, Cook et al., 1987). In phase I, bacteria on the seed attach to the emerging root tip and are passively transported into the soil. During root growth some cells remain associated with the tip while others are left behind on older portions of the root and in the rhizosphere. In phase II bacteria deposited along the root multiply and form microcolonies in nutrient-rich microsites, compete with indigenous microflora and avoid displacement. The concept of phase I and II is meant to define more easily two different stages, rather than a strict temporal sequence of events because both phases occur simultaneously on different parts of the roots (Weller and Thomashow, 1994). Some authors reported that bacterial cells first colonize the rhizosphere following soil inoculation (Gamalero, Lingua et al., 2009). Then, bacterial cells have been visualized as single cells attached to the root surfaces, and subsequently as doublets on the rhizodermis, forming a string of bacteria (Compant, Clément et al., 2010). Colonization may then occur on the whole surface and bacteria can even establish microcolonies or biofilms (Benizri, Baudoin et al., 2001). Nevertheless it is important to note that the root system is not colonized in

BOUMSI CENTRAL UNIVERSITY LIBRARY

a uniform manner, different population densities being reported for the diverse root zones. For example, *Kluyvera ascorbata* colonized the upper two-thirds of the surface of canola roots, but no bacteria were detected around root tips (Vessey, 2003). Non-uniform bacterial colonization along the root can be explained by different factors such as varying root exudation patterns, bacterial quorum sensing effects as well as many others (Compant, Clément et al., 2010).

Root colonization is influenced by many factors: biotic, such as genetic traits of the host plant and the colonizing organism or abiotic such as growth substrate, soil humidity, soil and rhizosphere pH, and temperature. This is why not any soil bacteria can colonize root system. In order to colonize the rhizosphere during an extended period characterized by strong microbial competition and exert plant growth promoting traits, soil bacteria need to be rhizosphere competent (Whipps, 2001). Root colonization and rhizosphere competence are heavily influenced by the plant changes in the physical and chemical composition of the rhizosphere soil compared to the bulk soil. These differences are manifested by changes in water potential, partial pressure of O_2 and other physical and chemical characteristics due to plant exudations (Vessey, 2003). There are several reports that link rhizosphere colonization to root exudation. Root exudates (carbohydrates, amino acids, organic acids) and mucilage-derived nutrients attract deleterious rhizobacteria as well as beneficial and neutral bacteria allowing them to colonize and multiply in the rhizosphere (Walker, Bais et al., 2003). Consequently PGPR have to be highly

BCU JASI / CENTRAL UNIVERSITY LIBRARY

competitive to successfully colonize the root zone (Compant, Clément et al., 2010).

Mechanisms of plant growth promotion

Investigations of the plant growth promotion mechanisms by PGPR are increasing at a rapid rate in order to use the best PGPR strain as commercial biofertilizer (Bhattacharyya and Jha, 2012). Many reports present, from different perspectives, the PGPR activities involved in plant growth enhancement. Further are presented the most cited mechanisms used by PGPR to promote plant growth.

As colonization occurs, PGPR interact with the plant host and enhance the nutrient uptake of host plants by (1) biological N_2 fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing increases in root surface area, (4) enhancing other beneficial symbioses of the host, and (5) combination of modes of action (Vessey, 2003). Some studies indicate that the effective PGPR increase plant growth basically by changing the whole microbial community structure in rhizosphere (Kloepper, Zablotowicz et al., 1991).

According to the mode of action, PGPR have been divided into two groups: PGPR that directly affect plant growth, seed emergence, or improve crop yields and biocontrol; PGPR that indirectly benefit the plant growth (Glick, 1999). Efforts have been made in the past two decades to elucidate both the direct and indirect mechanisms by which PGPR enhance plant growth (Podile and Kishore, 2006). The main mechanisms

BCU IASI CENTRAL UNIVERSITY LIBRARY

by which PGPR directly contribute to the plant growth are production of phytohormone like auxins, cytokinins and gibberellins, enhancement of plant nutrition by solubilization of minerals like phosphorus and iron, production of siderophores and enzymes, lowering of ethylene levels and induction of systemic resistance (Bhattacharyya and Jha, 2012). PGPR indirectly benefit the plant growth by the biocontrol of deleterious microorganisms or root pathogens that inhibit plant growth, including antibiotic production, parasitism, competition for nutrients and niches within the rhizosphere, synthesis of extracellular enzymes to hydrolyze the fungal cell wall, decreasing pollutant toxicity (Zahir, Arshad et al., 2003, Podile and Kishore, 2006, Bhattacharyya and Jha, 2012). Thus, based on their mechanism of action, PGPR can be categorized into three general forms such as *biofertilizer* (a substance that contains live microorganisms whith biological nitrogen fixation and phosphorus solubilization capabilities), *phytostimulator* (microorganisms with the ability to produce phytohormones) and *biopesticide* (microorganisms that promote plant growth by controlling phytopathogenic agents) (Bhattacharyya and Jha, 2012).

Biological N_2 fixation

Biological N_2 fixation is restricted to some prokaryotic organisms. The most studied and longest exploited PGPR are the rhizobia (including *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium*, and *Sinorhizobium* genera) for their ability to fix N_2 in their

BOUMERDES CENTRAL UNIVERSITY LIBRARY

legume hosts (Vessey, 2003). As these microorganisms may be autotrophic or heterotrophic, different strategies for garnering energy (particularly carbohydrates) have evolved in symbiotic rhizospheric-associations with plants as well as in free-living nitrogen-fixing organisms (Cocking, 2003). On average, symbiosis has the highest nitrogen fixation capability not only because energy (in the form of carbohydrates) is provided by the plant, but also due to other conditions (e.g., export of reduced nitrogen) optimized for efficient nitrogen fixation (Cocking, 2003). Symbiotic nitrogen fixation by rhizobia in legumes has a profound impact upon agriculture and human endeavour. This symbiosis involves intimate mutual interaction between both partners (plant and bacteria) with morphology alteration (Mukerji, Manoharachary et al., 2006). Because of the practical and theoretical importance of the symbiotic nitrogen fixation there are many review articles and monographs that cover this topic (Hansen, 1994, Rascio and La Rocca, 2008, Salvagiotti, Cassman et al., 2008).

Even if many PGPR present the ability to fix N_2, yet rarely is their mode of action for the stimulation of plant growth credited to biological N_2 fixation (Vessey, 2003).

Phytohormone production

By far the most evidence for the positive effects of PGPR points to bacteria-mediated changes in root growth and morphology (Vessey, 2003). With the production of different phytohormones like indole-3-acetic acid

BCU IASI CENTRAL UNIVERSITY LIBRARY

(IAA), gibberellic acid and cytokinins, PGPR can increase root surface and length and promote in this way plant development (Kloepper, Gutiérrez-Estrada et al., 2007). Several PGPR as well as some pathogenic, symbiotic and free living rhizobacterial species are reported to produce IAA and gibberellic acid in the rhizospheric soil and thereby plays a significant role in increasing the root surface area and number of root tips in many plants (Bhattacharyya and Jha, 2012). A greater root surface area enables the plant to access more nutrients from soil and thus contribute to plant growth promotion (Vessey, 2003).

IAA (auxin) is the most quantitatively important phytohormone produced by PGPR and treatment with auxin-producing rhizobacteria increases plant growth (Vessey, 2003). IAA is a phytohormone which is known to be involved in root initiation, cell division, and cell enlargement (Salisbury, 1994). Although different bacterial pathways used to synthesize IAA have been identified, a high degree of similarity between IAA biosynthesis pathways in plants and bacteria was observed (Spaepen, Vanderleyden et al., 2007). Tryptophan has been identified as a main precursor for IAA biosynthesis pathways in bacteria. The identification of intermediates led to the identification of five different pathways using tryptophan as a precursor for IAA: indole-3-acetamide pathway, indole-3-pyruvate pathway, tryptamine pathway, tryptophan side-chain oxidase pathway and indole-3-acetonitrile pathway (Spaepen, Vanderleyden et al., 2007).

Advancement in understanding the IAA signaling pathway in plants showed that the role of auxins in plant–microorganism interactions

BCU IASI / CENTRAL UNIVERSITY LIBRARY

appears diverse, varying from pathogenesis to phytostimulation. A number of studies have clearly shown that IAA can be a signaling molecule in microorganisms, in both IAA-producing and IAA-nonproducing species. These findings raise new intriguing questions on the role of IAA in bacteria and their interaction with plants (Spaepen, Vanderleyden et al., 2007).

Production of other phytohormones by biofertilizing-PGPR has been identified, but not nearly to the same extent as bacteria which produce IAA (Vessey, 2003). A few PGPR strains were reported to produce cytokinins and gibberellins (gibberellic acid) (Gutiérrez-Mañero, Ramos-Solano et al., 2001, Vessey, 2003). Other bacteria like *Azospirillum* and *Pseudomonas* spp. produce cytokinins and gibberellins, in addition to IAA (Gaudin et al. 1994). Cytokinins are a class of phytohormones which are known to promote cell divisions, cell enlargement and tissue expansion in certain plant parts (Zamfirache, 2005). Gibberellins are a class of phytohormones most commonly associated with modifying plant morphology by the extension of plant tissue, particularly stem tissue (Salisbury, 1994). However, the available information is insufficient to support the role of PGPR-produced cytokinins and gibberellins in plant growth promotion (Podile and Kishore, 2006).

Ethylene, a gaseous phytohormone commonly induced by wounding in plants, causes root growth inhibition. Studies of Glick et al. showed that many PGPR have the capability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme which

BCU IASI / CENTRAL UNIVERSITY LIBRARY

cleaves ACC, the immediate precursor of ethylene in the biosynthetic pathway for ethylene in plants (Glick, Penrose et al., 1998). In this way ACC producing PGPR can contribute to growth promotion even in stressful conditions such as flooded or cadmium contaminated soils (Podile and Kishore, 2006).

More recent discoveries of the involvement of IAA, cytokinins, ACC deaminase, and possibly gibberillins producing PGPR open the possibility that even more plant growth-regulating substances may be involved in the promotion of plant growth by some PGPRs (Vessey, 2003).

Phosphorous solubilization

Phosphorus (P), next to nitrogen, is one of the major macronutrients required for plant growth. Although the majority of agricultural soils contain large reserves of P accumulated as a consequence of regular applications of fertilizers most of the P is in insoluble form (iron and aluminium phosphates in acidic soils and calcium phosphates in alkaline soils) (Stevenson and Cole, 1999).

Several reports indicate that in soil and in plant rhizospheres are considerable populations of phosphate-solubilizing bacteria (PBS) (Bardiya, Gaur et al., 1974, Dey, 1988, De Freitas, Banerjee et al., 1997, Chen, Rekha et al., 2006). Different bacterial species present the ability to solubilize different insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock

BOJAS CENTRAL UNIVERSITY LIBRARY

phosphate. These bacteria occur in most soils and potentially represent 40% of the culturable population (Richardson, 2001). Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium* and *Erwinia* (Rodríguez and Fraga, 1999). The mechanisms involved in enhancing plant P-uptake by PBS in natural systems is not yet clearly elucidated (Mukerji, Manoharachary et al., 2006). PBS produces a range of organic acids such as citrate, lactate, and succinate that solubilise mineral phosphates. Gluconic acid and 2-ketogluconic acid appear to be the most active and important (Hwangbo, Park et al., 2003). Some Gram-negative rhizobacteria have membrane bound enzymes that enable the extracellular conversion of glucose to gluconic acid (glucose dehydrogenase) and thence to 2-ketogluconic acid (Mukerji, Manoharachary et al., 2006). Nevertheless, the organic acids released by plant roots probably have a greater impact on the solubilisation of P than do the acids produced by rhizobacteria (Jones, 1998). However, rhizobacteria consume the organic acids from root exudates and, therefore, indirectly may moderate solubilisation of P and other immobile elements, such as Fe and Mn (Mukerji, Manoharachary et al., 2006).

A wide range of organic substrates found in soil can also be an important source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P by means of acid and alkaline phosphatase enzymes. Because the pH of most soils ranges from acidic to neutral values acid phosphatases should play the major role

BCU IASI / CENTRAL UNIVERSITY LIBRARY

in this process (Rodríguez and Fraga, 1999). Approximately 30–48% of culturable soil and rhizosphere microorganisms utilize phytate (*myo*-inositol hexakisphosphate) (Greaves and Webley, 1965). Phytases were purified and characterized from selected soil bacteria including *Bacillus*, *Pseudomonas*, *Klebsiella* and *Enterobacter* spp. and are involved in the stepwise degradation of phytate to lower phosphate esters of myo-inositol and phosphorous (Podile and Kishore, 2006). All of these studies provide evidence that support the role of bacteria in rendering organic P available to plants. The possibility of enhancing P uptake of crops by artificial inoculation with P-solubilising strains of rhizobacteria presents an immense interest to agricultural microbiologists.

Siderophores production

Iron is an essential micronutrient for plants as it serves as a cofactor of many enzymes with redox activity and it is required in a number of major physiological processes like N_2-fixation, photosynthesis, respiration, etc. Under aerobic conditions, most iron exists in an insoluble form (Fe^{3+}) in soil, which is just not easily available to plants or microbes. To meet their iron requirement, microorganisms and plants have evolved specific mechanisms to chelate insoluble iron through the release of siderophores and uptake of iron-siderophore complexes through specific outer membrane receptor proteins (Sharma and Johri, 2003). These siderophores can be of different types: hydroxamates, phenol-catecholates, and carboxylates (Podile and Kishore, 2006). The synthesis of bacterial

BOUASI / CENTRAL UNIVERSITY LIBRARY

siderophores is induced by the low level of Fe^{3+}, and, in acid soil, where solubility and availability grow, their protective effect comes down. In such conditions, the efficiency of iron fixation can be raised by gaining mutant strains capable of synthesizing siderophores. This process is independent of iron concentration in soil solution (Maksimov, Abizgildina et al., 2011).

Some rhizospheric bacteria can produce siderophores and there is evidence that a number of plant species can absorb bacterial Fe^{3+}-siderophore complexes (Bar-Ness, Chen et al., 1991). However the significance of bacterial siderophore in the iron nutrition of plants is controversial (Vessey, 2003). Some authors believe that the contribution of these siderophores to the overall iron requirements of plants is small (Glick, 1995). Bar-Ness et al. concluded that two bacterial siderophores (pseudobactin and ferrioxamine B) were inefficient as iron sources for plants and that rhizospheric siderophore-producing bacteria can be in competition with the plant for iron (Bar-Ness, Hadar et al., 1992). Moreover microbial siderophores in the rhizosphere are frequently associated with biocontrol activities and not with plant nutrition (Vessey, 2003).

Biocontrol of plant diseases

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide (Compant, Brion et al., 2005). Diverse PGPR antagonize the root

BCU IASI / CENTRAL UNIVERSITY LIBRARY

pathogens through one or more different mechanisms, for example by production of bacterial allelochemicals such as volatile or non-volatile antibiotics, siderophores, detoxification enzymes, lytic enzymes and other secondary metabolites like HCN (Podile and Kishore, 2006). Production of these compounds is highly influenced by the qualitative and quantitative nutrient availability and is also subjected to quorum sensing (Haas and Keel, 2003).

Many PGPR present the ability to produce antibiotics. Some of these are oligopeptides that inhibit synthesis of pathogens cell walls, influence membrane structures of cells, inhibit the formation of initiation complex on small subunit of ribosomes (Maksimov, Abizgildina et al., 2011). A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol, hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads and oligomycin A, kanosamine, zwittermicin A, and xanthobaccin produced by *Bacillus*, *Streptomyces*, and *Stenotrophomonas* spp. (Compant, Brion et al., 2005). More than 12 antibiotics are synthesized by *B. subtilis* strains: bacillomicin, mycobacillin, fungistatin, iturin, fengicyn, plipastatin, surfactin, bacilizin, etc. The majority of *Bacillus* spp. antibiotics are active against both Gram positive and Gram negative bacteria (for example, polymyxin, circulin, and colistin) and pathogenic fungi *Alternaria solani, Aspergillus flavus, Botryosphaeria ribis, Colletotrichum gloeosporioides, Fusarium oxysporum, Helminthosporium maydis*, *Phomopsis gossypii* (Maksimov, Abizgildina et al., 2011). Some studies reported an active influence of

BCU ASI CENTRAL UNIVERSITY LIBRARY

bacterial antibiotics in the regulation of defense system of the plant. It was revealed that *B. subtilis* surfactine is able to stimulate induced systemic resistance by activation of components like lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (Ongena, Jourdan et al., 2007, Maksimov, Abizgildina et al., 2011).

Certain PGPR strains produce volatile antimicrobial substances of which HCN that inhibit the cytochrome oxidase of many organisms is most important. The producer strains possess an alternate cyanide-resistant cytochrome oxidase and are relatively insensitive to HCN (Podile and Kishore, 2006).

Siderophores produced by PGPR inhibit the root pathogens by creating iron limiting conditions in the rhizosphere and reduces probability of plant disease (Podile and Kishore, 2006). Some siderophores such as pseudobacin and pyoverdin (yellow green fluorescent pigment of *Pseudomonas* bacteria) present high antimicrobial activity and affinity to ions of trivalent iron (Maksimov, Abizgildina et al., 2011). Pseudobacin is involved in induced systemic resistance, induction of H_2O_2 local storage, and strengthening cell wall of rice plants in infection zone. Pyoverdin is one of the most important antimicrobial compounds in *Pseudomonas putida* (Maksimov, Abizgildina et al., 2011). However, the suppression of pathogens by majority of the siderophore-producing PGPR is due to a combination of various other traits (Ongena, Daayf et al., 1999).

Some bacteria, especially *Bacillus* and *Pseudomonas* spp. depress growth and development of filamentous fungi both *in vitro* and *in vivo* by

BCU INSTITUTE CENTRAL UNIVERSITY LIBRARY

secreting lytic enzymes such as chitinases and gluconases. It is assumed that bacteria producing chitinases could be used for biological protection of crops from pathogens, especially those that contain chitin and glucans within their cell wall structure (Maksimov, Abizgildina et al., 2011).

Interestingly, some allelochemicals produced by PGPR are finding new uses as experimental pharmaceuticals, and this group of bacteria may offer a resource for compounds to deal with the alarming ascent of multidrug-resistant human pathogenic bacteria (Compant, Brion et al., 2005).

Combination of mechanisms

Some PGPR can induce plant growth promotion through a combination of action modes. Studies concerning PGPR screening showed that in fact in many cases (possibly most cases) a single PGPR will display several traits (Vessey, 2003). Dey et al. (2004) reported that besides ACC deaminase activity, expression of one or more of the traits such as suppression of phytopathogens, solubilization of tricalcium phosphate, production of siderophore and/or nodulation promotion by the PGPR might have simultaneously contributed to the enhancement of growth, yield, and nutrient uptake of peanut (Dey, Pal et al., 2004, Khan, Zaidi et al., 2009).

Isolation of bacteria from rhizosphere will often reveal multiple modes of action from the population of putative PGPR inhabiting the rhizosphere (Vessey, 2003). For example Antoun et al. (1998) isolated

BCU JAISI CENTRAL UNIVERSITY LIBRARY

266 strains of rhizobia and found 83% produced siderophores, 58% produced IAA and 54% could solubilize phosphorus (Antoun, Beauchamp et al., 1998). Belimov et al. (2001) investigated 15 strains of bacterial isolated from the rhizoplane of pea and Indian mustard and found that of five *Pseudomonas* isolates three were positive for ACC-deaminase and phosphate solubilization activity, and two were positive for ACC deaminase activity, phosphate solubilization activity and IAA production (Belimov, Kojemiakov et al., 1995). From these studies it can be hypothesized that PGPR co-existing in the rhizosphere that have single modes of action may act synergistically to stimulate the growth of the host plant (Vessey, 2003)

Since the term was first introduced in the late 1970s, the research in PGPR has gain momentum, in the last decades new papers on this subject have been published at an ever increasing rate. This scientific interest is related to the PGPR potentiality in agriculture which is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. Conventional farming practices that warrant high and qualitative yield require intensive use of chemical fertilizers, but which are costly and have a high pollution effect (Orhan, Esitken et al., 2006). Therefore, more recently there has been a resurgence of interest in environmental friendly, sustainable and organic agricultural practices (Esitken, Pirlak et al., 2006). To the maximum extent feasible, organic agricultural systems rely upon bio-fertilization, crop rotations, crop residues, animal manures, legumes, green manures,

BCU IASI / CENTRAL UNIVERSITY LIBRARY

off-farm organic wastes and mineral-bearing rocks to maintain soil productivity. Given the negative environmental impact of chemical fertilizers and their increasing costs, the use of PGPR is advantageous in the sustainable agricultural practices (Chen, Rekha et al., 2006). Recent progress in our understanding on the diversity of PGPR in the rhizosphere along with their colonization ability and mechanism of action should facilitate their application as a reliable component in the management of sustainable agricultural system (Bhattacharyya and Jha, 2012).

Biotechnology has opened up new possibilities concerning the application of beneficial bacteria in the soil for the promotion of plant growth and the biological control of soil-borne pathogens (Kloepper, Lifshitz et al., 1989). Since the large scale release of genetically engineered bacteria to the environment faces a number of regulatory hurdles, the need to isolate and select superior, naturally occurring rhizosphere bacteria continues to be of interest (Bashan, Holguin et al., 1993). The utilization of rhizobacteria in agricultural production depends on our knowledge about bacteria–plant interaction and our ability to maintain, manipulate and modify beneficial bacterial populations under field conditions (Hammer, Carson et al., 1999).

Identification of different mechanisms involved in plant-rhizosphere microorganism interaction opened new possibilities to design strategies for improving crop yields. The literature describes many beneficial effects of PGPR on plant growth from changing the root architecture and enhancing nutrient uptake to biocontrol. Along this, biotechnology can be applied to further improve strains that have PGPR

BCUJASH CENTRAL UNIVERSITY LIBRARY

qualities in order to create transgenic strains that combine multiple mechanisms of action. Despite all this, a new question arises: *is the modification of plant rhizosphere by PGPR artificial introduction beneficial to normal plant processes and to natural root microbiota structure*? Our understanding of plant-microbe interaction in rhizosphere must increase before we can presume that utilization of PGPR as biofertilizers will determine a sustainable promotion of host plants growth.

1.1. SCREENING FOR PLANT GROWTH PROMOTING RHIZOBACTERIA

Soybean (*Glycine max* L.) and runner bean (*Phaseolus coccineus* L.) are two of the the most important legume crops in the world. Both species present many important features that can make it quite suitable for sustainable agricultural systems, including organic systems. Therefore the developing of biofertilizers for crop production is a practical necessity.

1.1.1. Characterization of bacterial strains isolated from soybean rhizosphere

A number of 26 strains were isolated from the rhizosphere and rizoplane of field-grown soybean (Ezareni, Iaşi county) based on differences in colony morphology including: colony form, elevation, opacity and pigment production. The selected strains were further tested

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

for mineral phosphate solubilization, siderophore and IAA production in order to assess their plant growth promoting capabilities.

Phosphorous solubilization

In the areas surrounding soybean roots is developing a morphologically less diversified bacterial population represented predominantly by sporulated and non-sporulated Gram positive rods, also by cocci and cocobacili - Fig. 1 (Apostu, Petriman et al., 2010).

The morphological structure of the soybean rhizosphere microbiota largely corresponds with the data reported in the literature (Lynch, 1990, Liu and Sinclair, 1993, Sylvia, Fuhrmann et al., 1999).

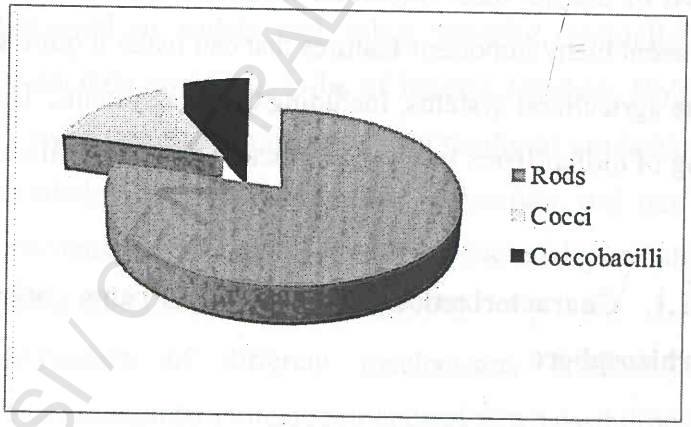


Fig. 1 – The main morphological bacterial types isolated from soybean roots

Qualitative screening of the phosphate solubilizing microorganisms was possible using a plate method (De Freitas, Banerjee

BCU IASI / CENTRAL UNIVERSITY LIBRARY

et al., 1997). In this case clearing zones appear around the microbial colonies in media containing insoluble mineral phosphates as single P source (Fig. 2). From the total number of isolates 31.03% presented P solubilizing capabilities.

Fig. 2 – Qualitative screening of the phosphate solubilizing rhizobacteria: Rzp4 strain

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986, Richardson, 1994, Singh and Kapoor, 1999). There are many populations of P solubilizing bacteria in soil, but considerably higher concentration of these microorganisms is commonly found in the rhizosphere (Katznelson, Peterson et al., 1962, Raghu and

BCU IASI CENTRAL UNIVERSITY LIBRARY

MacRae, 1966, Laheurte and Berthelin, 1988, Richardson, 1994). In the case of soybean, the majority of the strains with P solubilizing capabilities were isolated from rhizosphere and not from the free soil.

The presence of P solubilizing bacteria in soybean rhizosphere may be correlated with the special nutritional needs of this plant and with the complex interrelations that are set in the root zone. Thus, it is considered that phosphorus promotes development of soybean nitrogen fixing nodules by stimulating the development of nitrogen-fixing bacteria via a mechanism that includes increased P availability (De Freitas, Banerjee et al., 1997). Considering that P availability is a limiting step in plant nutrition (Goldstein, 1986), this evidence suggests a fundamental contribution of phosphate-solubilizing bacteria to plant nutrition and to the improvement of plant growth performance (Rodríguez and Fraga, 1999); therefore it could be considered that the strains isolated from soybean rhizosphere present a good capacity to be used in further experiments as PGPR.

Siderophores production

The 26 isolates were tested for siderophores producing capabilities based on the O-CAS assay (Schwyn and Neilands, 1987). A number of 17 strains were found positive for siderophore production. There were no significant qualitative differences among strains isolated from rhizosphere and from rizoplane, although most of the positive strains were isolated from the rhizospheric soil (10 strains) – (Achitei, Stefan et al., 2010).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Several microorganisms produced changes in medium coloration that are in agreement with previously reported studies (Baakza, Vala et al., 2004, Perez-Miranda, Cabirol et al., 2007). Hence, strain R2.7 changed the color to orange (which corresponds to hydroxamates, as mentioned above), whereas other strains changed the color to purple (which corresponds to catechol-type siderophores) - Fig. 3 and Table 1.

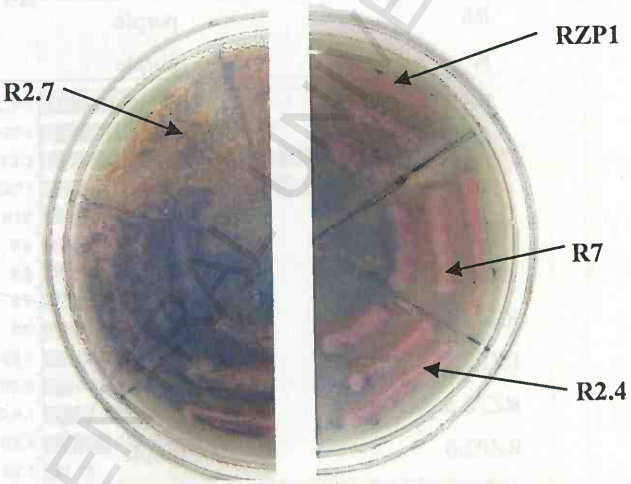


Fig. 3 – O-CAS assay performed using different rhizobacterial strains. Different changes in the color of the medium can be appreciated: left – R2.7 strain that produce hydroxamates; right - strains that produce siderophores (catechol type)

BCU IASI CENTRAL UNIVERSITY LIBRARY

Table 1 – Siderophores production by soybean rhizobacterial isolates

Strains	Results	O-CAS medium color
R1	+	purple
R2	-	-
R2.1	+	purple
R2.3	-	-
R2.4	+	purple
R2.5	+	purple
R2.6	+	purple
R2.7	+	orange
R6	+	purple
R7	+	purple
R8	+	purple
R9	-	-
R10	+	purple
RZP1	+	purple
RZP2	-	-
RZP2.1	+	purple
RZP2.3	+	purple
RZP2.5	-	-
RZP2.6	+	purple
RZP3	-	-
RZP4	+	purple
RZP5	+	purple
RZP6	+	purple
RZP8	-	-
RZP9	-	-
RZP10	-	-

+: positive reaction

-: negative reaction

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Production of Indoleacetic Acid

The selection of indoleacetic acid producing microorganisms was based on colorimetric IAA assay (Gordon and Weber, 1951). Only 13 strains were found positive for IAA production with different IAA synthesis capabilities (Achitei, Stefan et al., 2010). The vast majority of the positive strains were isolated from the soil surrounding soybean roots (9 strains), although the best IAA producers were found in the rizoplane (RZP2.3 – 3.92 µg IAA/ml and RZP1 – 3.14 µg IAA/ml) – Fig. 4.

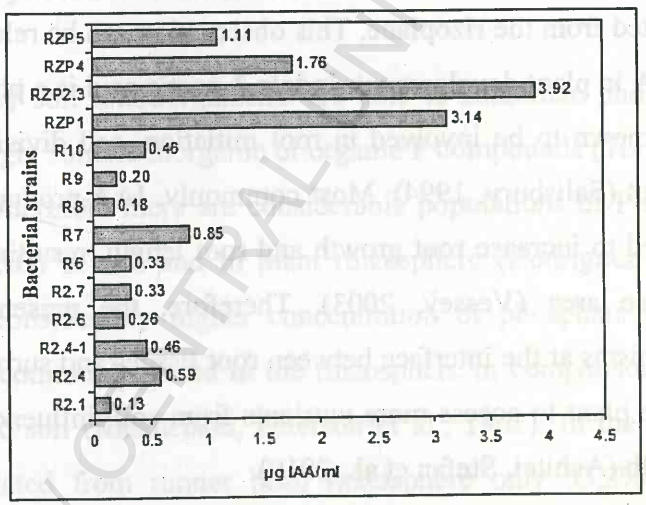


Fig. 4 – IAA producing capabilities of different bacterial strains isolated from soybean roots

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms (Vessey, 2003).

BCU IASI CENTRAL UNIVERSITY LIBRARY

These mechanisms includes siderophores and phytohormones production (Bashan, 1998, Saleh and Glick, 2001). More than 65% of the bacterial strains isolated from soybean roots produced siderophores of different types (mainly catechol and hydroxamates type) as well as more than 50% presented IAA producing capabilities. Most of these strains were isolated from the rhizosphere. This is not an unusual situation since rhizosphere is defined as the soil directly influenced by root secretions with intensive microbial activities (Lynch, 1990). Although the rhizosphere can be a great source of bacteria with plant growth promoting potential (Zahir, Arshad et al., 2003), the strains with the highest IAA producing capability were isolated from the rizoplane. This observation can be related with the role of IAA in plant development. Indole-3-acetic acid is a phytohormone which is known to be involved in root initiation, cell division, and cell enlargement (Salisbury, 1994). Most commonly, IAA-producing bacteria are believed to increase root growth and root length, resulting in greater root surface area (Vessey, 2003). Therefore, the presence of such microorganisms at the interface between root tissues and surrounding soil enables the plant to access more nutrients from soil, influencing directly plant growth (Achitei, Stefan et al., 2010).

In normal conditions, it is not uncommon to see the promoting plant growth effects of rhizobacteria via a synergism of the rhizospheric microorganism (Vessey, 2003). The same situation occure in soybean rhizosphere since more than 12 bacterial strains isolated from rhizosphere and rizoplane presented both siderophores and IAA producing capabilities.

BCU MASTER CENTRAL UNIVERSITY LIBRARY

1.1.2. Characterization of bacterial strains isolated from runner bean rhizosphere

A number of 24 rhizobacteria isolates from runner bean rhizosphere were selected to represent distinct types based on differences in colony morphology (Stefan, Munteanu et al., 2013). In order to assess their plant growth promoting capabilities the selected strains were tested for mineral phosphate solubilization, IAA and siderophore production.

Phosphorous solubilization

Many soil microorganisms are able to solubilize phosphate ions from sparingly soluble inorganic or organic P compounds (Toro, Azcón et al., 1996), therefore there are considerable populations of P-solubilizing bacteria (PSB) in soil and in plant rhizosphere (Rodríguez and Fraga, 1999). A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with non-rhizospheric soil (Katznelson, Peterson et al., 1962). In the case of the strains isolated from runner bean rhizosphere only 33.3% of the 24 rhizobacteria isolates solubilized $Ca_3(PO_4)_2$ in the qualitative plate method. Some obvious differences in the size of the halos of different isolates were observed (Table 2).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Table 2 – P solubilization, IAA and siderophores production by rhizobacteria isolates (n=9)

Strains	P solubilization (mm)[1]	IAA-like compounds (µg IAA/ml)[2]	Siderophores production	
			Results	O-CAS medium color
S1	-	0.19	+	purple
S2	2.75 ± 0.80	nd	-	-
S3	1.54 ± 1.3	nd	-	-
S4	4.9 ± 1.22	nd	+	purple
S5	-	nd	-	-
S6	-	1.19	-	-
S7	-	4.86	-	-
S8	-	0.64	-	-
S9	-	3.81	-	-
S10	3.91 ± 0.87	nd	-	-
S11	-	1.15	+	purple
S12	-	nd		
S13	-	nd	+	purple
S14	-	nd	-	-
S15	2.30 ± 0.9	nd	+	purple
S16	-	0,97	-	-
S17	1.18 ± 1.6	0.28	+	orange
S18	-	nd	+	purple
S19	-	nd	-	-
S20	2.86 ± 0.97	0.61	+	
S21	-	1.42	-	-
S22	-	0.97	-	-
S23	1.91 ± 2.4	nd	+	orange
S24	-	2.74	-	-

BCU IASI / CENTRAL UNIVERSITY LIBRARY

[1] Results represent the mean values ± SD of the ratio between the diameter of the halo and the diameter of the colony (mm)

[2] Production of IAA-like compounds determined in liquid medium amended with L-tryptophan after 72 hours growth (Gordon and Weber, 1951)

nd – not detected

+: positive reaction

-: negative reaction

Similarly percentages of PSB presence in the rhizosphere of different plants (wheat, pea, canola, barley, and corn) was also reported (De Freitas, Banerjee et al., 1997). PSB are considered to play an important ecophysiological role in mobilizing insoluble inorganic phosphates from their mineral matrix to the bulk soil where they can be absorbed by plant roots (Pérez, Sulbarán et al., 2007). Therefore the PSB isolate with the biggest ratio between the diameter of the halo and the diameter of the colony (mm) was named S4 and was selected for further studies concerning development of runner bean plants.

The best PSB isolates were selected for further studies concerning P-solubilization in liquid culture (Mihalache, Munteanu et al., 2012). All these isolates showed higher potential for solubilization of inorganic P as indicated by the increase of P amount in the culture medium supplemented with $Ca_3(PO_4)_2$ (Fig. 5).

The amount of solubilized P varied from 7.91 µg P/ml to 13.46 µg P/ml. The highest dissolved P value was exhibited by S6 strain (13.46 µg P/ml), followed by S20 (11.48 µg P/ml), S10 (11.27 µg P/ml) and S3 (11.23 µg P/ml). Most of the tested isolates showed significant differences

BCU IASI CENTRAL UNIVERSITY LIBRARY

in the amount of solubilised P versus uninoculated control ($p<0.05$), except strain S19 ($p<0.06$).

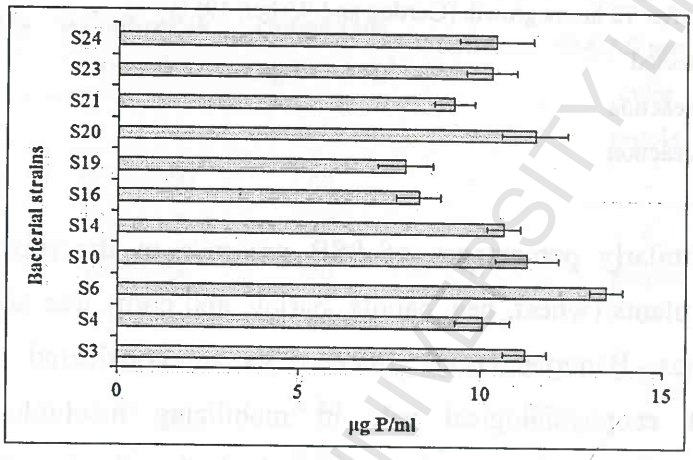


Fig. 5 – P solubilized from calcium phosphate by rhizobacteria grown in liquid cultures (n=3)

All the rhizobacterial isolates decreased the pH value of the growth medium from the initial value of 7.03 ± 0.05 to a pH value of 5.13 ± 0.025 (Fig. 6).

Mechanisms of phosphorous solubilization

It is generally accepted that the main mechanism of mineral phosphate solubilization is represented by the production of organic acids (gluconic and lactic acid), as well as inorganic acids, such as sulphidric nitric, and carbonic acid (Rodríguez and Fraga, 1999). The main strategy

BCU IASI CENTRAL UNIVERSITY LIBRARY

used by the PSB isolated from runner bean rhizosphere to solubilize inorganic P seemed to be acidification of the growth medium. The results showed a clear correlation between bacterial growth, supernatant acidification and P solubilization. An inverse relationship between the pH and soluble-P concentration was observed (Fig. 6). This may indicate that acidification of the culture medium might be due to the production and excretion of organic acids, which may also act as chemical agents for mobilizing insoluble phosphates (Pérez, Sulbarán et al., 2007).

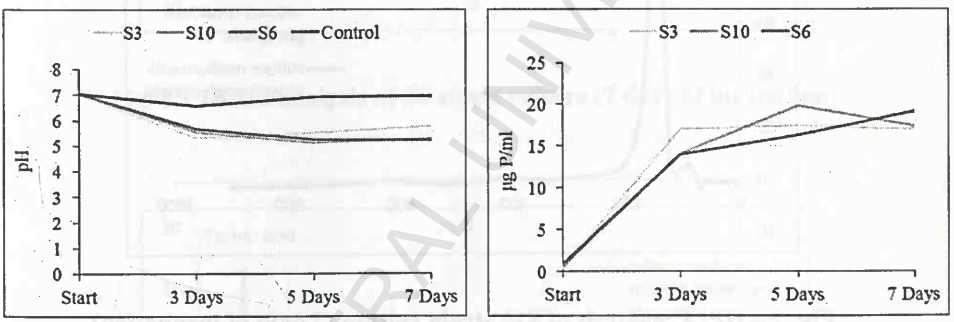


Fig. 6 – Changes in pH value (left) and P-solubilization (right) during the 7 day incubation in PVK broth

Similar inverse relationship between pH and soluble phosphate was reported earlier by other authors (Illmer, Barbato et al., 1995, Hwangbo, Park et al., 2003, Chen, Rekha et al., 2006). Also it has been well established that, as a common strategy to scavenge P from insoluble mineral sources, microorganisms produce and exudate organic acids and/or chelating agents (Rodríguez and Fraga, 1999). Therefore a

BCU IASI CENTRAL UNIVERSITY LIBRARY

chromatographic analysis of the culture supernatants (PVK broth supplemented with tricalcium phosphate) was performed. The results obtained for different strains showed a significant accumulation of isocitric acid (Fig. 7), lactic acid (Fig. 8), and tartaric acid (Fig. 9) compared with control.

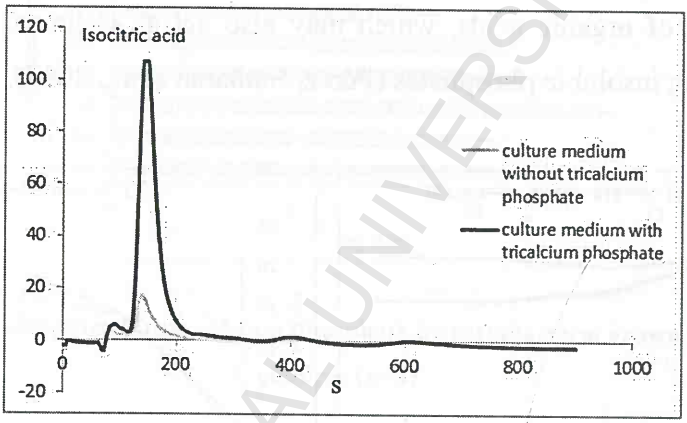


Fig. 7 – HPLC analysis of S10 strain culture (7 days of incubation)

Several authors proposed as another mechanism involved in mineral phosphate solubilization the phosphatases activity (Ponmurugan and Gopi, 2006, Pantujit and Pongsilp, 2010, Park, Bolan et al., 2011). All tested strains exhibited high levels of acid and alkaline phosphatase activity in the medium with tricalcium phosphate (at least in the 7th day of incubation) – Fig. 10. Also P solubilization appeared to be related to acid and alkaline phosphatase activity, the highest amount of P in cultures was recorded when the enzymes activity levels were maximum (days 5 and 7).

BCU IASI CENTRAL UNIVERSITY LIBRARY

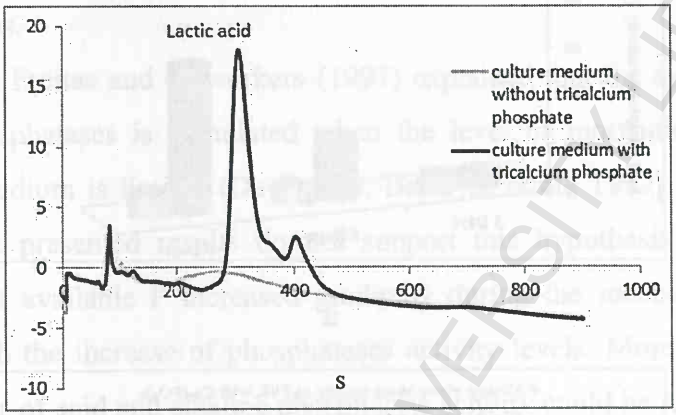


Fig. 8 – HPLC analysis of S6 strain culture (7 days of incubation)

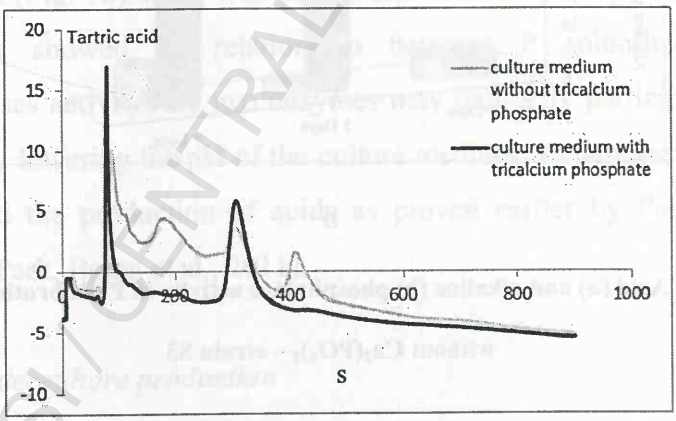
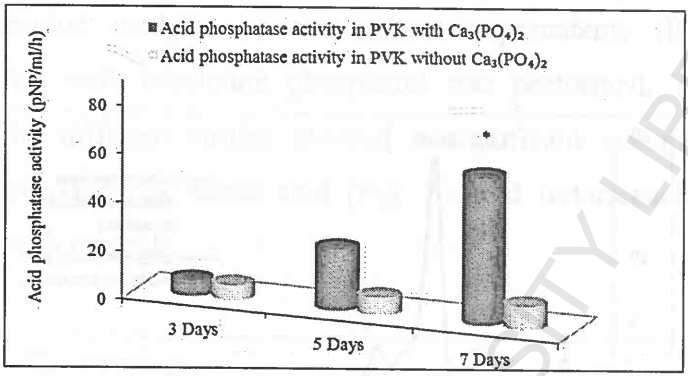
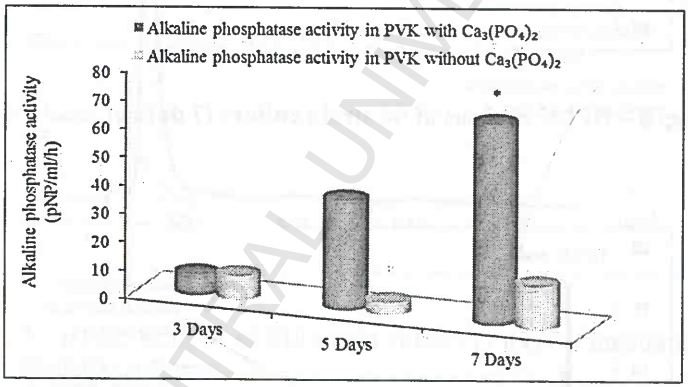


Fig. 9 – HPLC analysis of S20 strain culture (7 days of incubation)

BCU IASI / CENTRAL UNIVERSITY LIBRARY



a



b

Fig. 10 - Acid (a) and alkaline (b) phosphatase activity in PVK broth with and without $Ca_3(PO_4)_2$ – strain S3

However, this apparent relationship between P-solubilization and phosphatase production is misleading because no significant positive correlation between the amount of solubilized phosphorus and the level of acid and alkaline phosphatase activity could be inferred. So, it can be

BCU IASI / CENTRAL UNIVERSITY LIBRARY

presumed that the two enzymes are not involved in the mechanisms used by these isolates from the runner bean rhizosphere to solubilize inorganic phosphorus.

De Freitas and co-workers (1997) explained that the synthesis of these phosphatases is stimulated when the level of inorganic-P in the growth medium is limited (De Freitas, Banerjee et al., 1997). However, the above presented results do not support this hypothesis since the amount of available P increased gradually during the incubation time, along with the increase of phosphatases activity levels. More likely the high levels of acid and alkaline phosphatase activity could be related with the culture growth. For instance the data recorded for S3 strain showed that the phosphatases activity gradually increased during the 7 days of incubation (Fig. 10) along with the optical density of the culture. Even if the study showed no relationship between P solubilization and phosphatases activity, the two enzymes may indirectly participate to this process by lowering the pH of the culture medium via dephosphorylating action and the production of acids as proven earlier by Park and co-workers (Park, Bolan et al., 2011).

Siderophore production

Rhizobacterial isolates were assessed for siderophore production on Chrome azurol S agar medium, as described by Schwyn and Neilands (Schwyn and Neilands, 1987). Production of siderophore was exhibited by 37.5% of the tested strains. Most of these strains produced catechol-

BCU AS / CENTRAL UNIVERSITY LIBRARY

type siderophores (a change in the dye color from blue to purple was observed). Only two strains (S17 and S23) changed the medium color to orange which corresponds to hydroxamates - Table 2 (Stefan, Munteanu et al., 2013).

Production of Indoleacetic Acid

Production of the phytohormone IAA is widespread among bacteria that inhabit the rhizosphere of plants (Patten and Glick, 1996). Detection of IAA producing bacteria was based on a colorimetric assay. The results presented in Table 2 indicate that different rhizobacteria isolates varied greatly in their efficiency of producing auxins in liquid medium amended with L-tryptophan after 72 h growth. Half of the 24 tested rhizobacterial strains produced auxins in the medium to a concentration ranging from 0.19 to 4.86 μg IAA/ml (Stefan, Munteanu et al., 2013). The best IAA yield was recorded for strain S7 isolated from soybean rhizosphere, which was selected for further field studies using runner bean plants.

A number of 24 rhizobacteria isolates were screened *in vitro* for plant growth promoting (PGP) activities. Some of the tested strains exhibited more than one PGP trait which may promote the growth directly, indirectly or synergistically. Similar results concerning multiple PGP activities among PGPR were previously reported by other authors (Gupta, Singal et al., 1994, Ahmad, Ahmad et al., 2008). Based on the preliminary screening test, strains S4 (positive for phosphate solubilization and

BCU IASI / CENTRAL UNIVERSITY LIBRARY

siderophore production) and S7 (positive for IAA production) were selected for further field studies in order to assess their plant growth promoting potential for runner bean plants. Because at the present time little is known about the PGPR's interactions in the rhizosphere, the experiments were carry out using a single strain (S4 or S7) or a combination of strains (S4+S7) to establish which version is more suitable to enhance runner bean plants growth.

1.2. RHIZOBACTERIA USED AS BIOFERTILIZERS

1.2.1. Plant growth promoting rhizobacteria can inhibit the germination of soybean seeds

The interaction between different rhizobacteria strains with soybean seeds was studied during the *in vitro* germination process (Stefan, Mihăşan et al., 2008). The activity of two key enzymes involved in protein metabolism (aspartate- and alanin-aminotransferase) and one biologic stress marker (superoxide-dismutase) was assessed.

Soybean seeds are very rich in proteins, these bio-molecules being one of the main resources which must be mobilized during the germination process. Utilization of protein as C and N sources requires the presence of proteolytic enzymes and aminoacid transferases such as aspartate- and alanin-aminotransferase. These enzymes are necessary in order to link the protein metabolism to all the other metabolic pathways in

BCU IASI CENTRAL UNIVERSITY LIBRARY

the cell. This is why the activity of the two above mentioned enzymes must be considered a key indicator for the protein metabolism in the germination process under the influence of PGPR. Previous observations showed that the germination process is slowed down *in vitro* conditions by the presence of PGPR – (Stefan, Mihăşan et al., 2008). This could be due to a stressing effect induced by PGPR or to a competition for nutrient resources between plant and inoculated rhizobacteria. In order to answer this question the activity of aspartate-aminotransferase, alanin-aminotransferase and SOD were measured in several points during *in vitro* germination.

The evolution of aspartate-aminotransferase activity during the *in vitro* germination can be observed in Fig. 11.

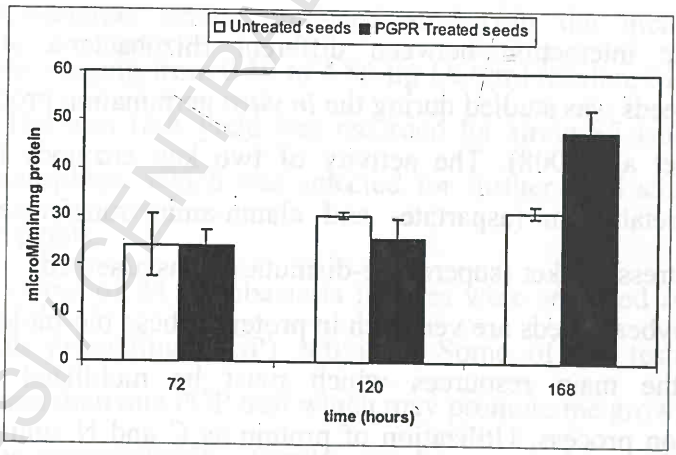


Fig. 11 – Aspartat-aminotransferase activity during the germination of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

BCU IASI / CENTRAL UNIVERSITY LIBRARY

In the first stages of germination (72 hours), both the untreated and PGPR treated seeds express the same level of activity for this enzyme. At 120 hours from inoculation, the level of aspartate-aminotransferase activity is higher in the un-inoculated seeds (30.38 ± 0.672 microM/min/mg protein) compared to PGPR treated ones (25.46 ± 4.12 microM/min/mg protein). This difference is significant ($p=0.03$) and shows that the proteins are faster metabolized in the case of un-treated seeds. During the germination, the seeds have to produce from proteins different non-proteic compounds (sugars for cell walls, lipids for membranes, etc) before the photosynthetic processes begins, so the recorded delay in protein metabolism of PGPR treated seeds would lead to a decrease of the germination processes.

This hypothesis is also sustained by the level of aspartate-aminotransferase activity recorded at 168 hours. At this point, the PGPR treated seeds show a higher activity of this enzyme (47.92 ± 4.58 microM/min/mg protein) compared with untreated seeds (31.17 ± 1.282 microM/min/mg protein). The significant differences ($p=0.0005$) show that untreated seeds are approaching to the end of the germination, fact proved also by the development of the first green leaves. The PGPR treated seeds show a more intense protein metabolism, as they are in the earlier stages of germination (at 168 hours the green leaves are not fully developed and thereby photosynthesis cannot occure).

The evolution of alanin-aminotransferase activity during the germination of soybean seeds demonstrates the same PGPR inhibitory effect (Fig. 12).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

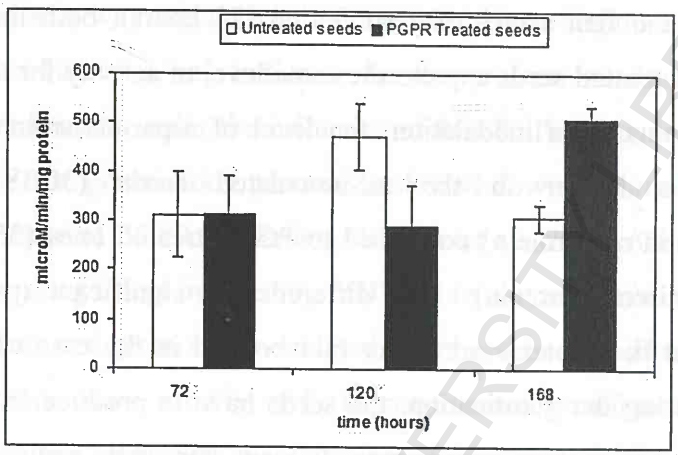


Fig. 12 – Alanin-aminotransferase activity during the germination of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

So, if at 72 hours the recorded differences between the enzymatic activities were not significant, at 120 hours the untreated seeds show a more active protein metabolism (471.94 ± 67.32 microM/min/mg protein) compared with the PGPR treated ones (288.27 ± 83.98 microM/min/mg protein). The recorded differences are significant (p=0.001).

These results presented so far are in good acordance with previous studies regarding the total soluble protein content evolution during the *in vitro* germination of soybean seeds (Mihăşan and Ştefan, 2008). The reported delay in the germination of PGPR treated seeds can be explained taking into account two possible aspects. First of all the inhibitory effect could be the result of an oxidative stress induced by the presence and activity of PGPR used for seed inoculation. The second possible explanation is a competition for nutrient resources between soybean seeds

BCU IASI CENTRAL UNIVERSITY LIBRARY

and rhizobacteria. In order to establish the cause of the germination delay an oxidative stress marker enzyme was studied: superoxide-dismutase (SOD) - Fig. 13.

Although apparently an increase in SOD activity can be observed during the germination processes for both untreated and PGPR treated seeds, no statistically significant differences were recorded. This would indicate that the oxidative stress is at the same level for test and control disregarding the presence of PGPR. In the case of PGPR treated seeds the germination is inhibited due to a nutrient competition between inoculated rhizobacteria and soybean seeds. *In vitro* condition the seeds were hydrated using distillated sterile water and the support was plain sterile filter paper. This means that the only nutrient source for rhizobacterial growth remains the soybean seeds.

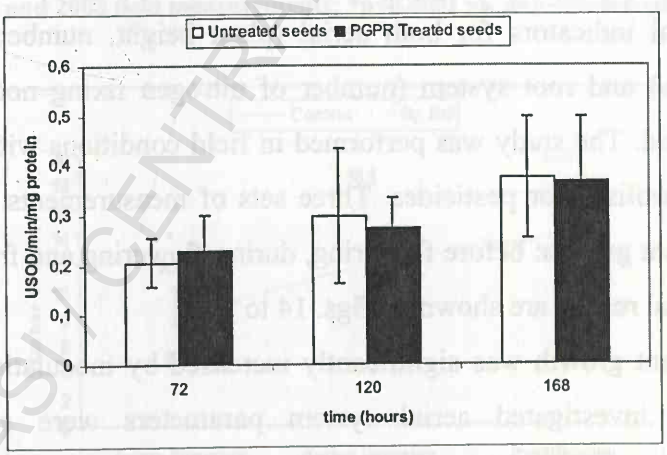


Fig. 13 – SOD activity during the germination of *Glycine max* L. seeds (white – untreated seeds, black - PGPR treated seeds)

BCUIASI / CENTRAL UNIVERSITY LIBRARY

1.2.2. Soybean inoculation with *Bacillus pumilus* Rs3 promotes plant growth and increases seed protein yield

In the selection of plant growth promoting bacteria the final evaluation must be made under field conditions. Compared to a controlled environment as a greenhouse, the field environment is generally much more stressful and the conditions are much more complex. Thus, results obtained in greenhouse experiments do not necessarily reflect the potential for plant growth promotion in the field (Khan, Zaidi et al., 2009). Therefore one *Bacillus pumilus* strain (Bp Rs3 - isolated from soybean rhizosphere, with phosphorous solubilizing capabilities) was used for field growth experiments (Stefan, Dunca et al., 2010).

In order to evaluate the plant growth promotion effect some biometrical indicators for both aerial (plant height, number of leaves, foliar area) and root system (number of nitrogen fixing nodules) were investigated. The study was performed in field conditions without use of organic fertilizers or pesticides. Three sets of measurements were made during plant growth: before flowering, during flowering and fructification. Biometrical results are shown in Figs. 14 to 16.

Plant growth was significantly increased by inoculation with Bp Rs3. All investigated aerial system parameters were significantly increased compared with non-treated control: plant height ($p<0.0001$), number of leaves ($p<0.0003$), foliar area ($p<0.0002$). Also, Bp Rs3 significantly increased the nitrogen fixing nodules number per plant

BCU JASSY CENTRAL UNIVERSITY LIBRARY

(p<0.0002) and promoted root system growth compared with non-treated control.

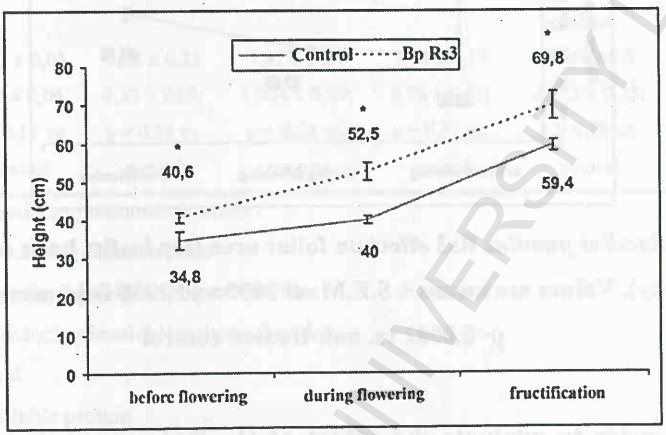


Fig. 14 – *Bacillus pumilus* Rs3 effect on plant height. Values are means ± S.E.M. of 2007 and 2008 field measurements; *p<0.0001 vs. non-treated control

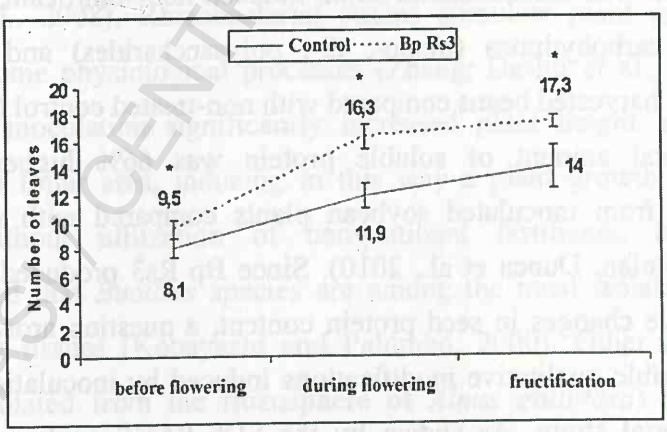


Fig. 15 – *Bacillus pumilus* Rs3 effect on intact trifoliate leaves number. Values are means ± S.E.M. of 2007 and 2008 field measurements; * p<0.0003 vs. non-treated control

BCU IASI CENTRAL UNIVERSITY LIBRARY

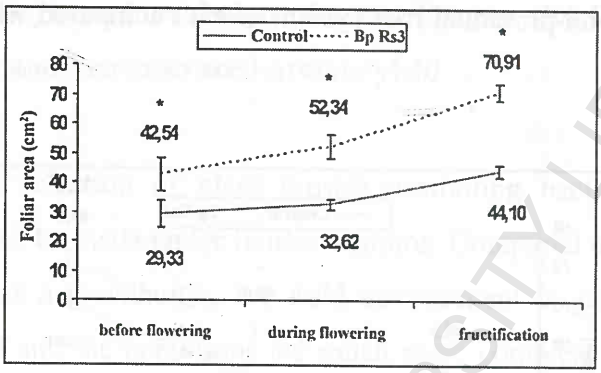


Fig. 16 – *Bacillus pumilus* Rs3 effect on foliar area (top leaflet basis using digital photography). Values are means ± S.E.M. of 2007 and 2008 field measurements; * $p<0.0002$ vs. non-treated control

In order to evaluate the effect of Bp Rs3 on nutritional value of harvested beans the chemical composition of soybean seeds was analyzed by assessing total soluble proteins, total lipid and carbohydrates content. Inoculation with rhizobacterial strain induced non-significant increase of reducing carbohydrates (mono-, di-, polysaccharides) and total lipid content in harvested beans compared with non-treated control (Table 3).

Total amount of soluble protein was 66% higher in beans harvested from inoculated soybean plants compared with non treated control (Stefan, Dunca et al., 2010). Since Bp Rs3 produced significant quantitative changes in seed protein content, a question arose regarding some possible qualitative modifications induced by inoculation with the rhizobacterial strain. As shown by the SDS-PAGE analysis (Fig. 17), inoculation of soybean plants with Bp Rs3 did not induced any detectable qualitative modifications in the seed proteins electrophoretic pattern.

BUAIASI CENTRAL UNIVERSITY LIBRARY

Table 3. Effects of inoculation with Bp Rs3 on chemical composition of harvested beans

Group	TRM	TRD	TRSP	TRIP	TL	TSP
	g/100 g beans				g/100 dried beans	g BSA/100 g beans
Control	0,57 ± 0,03	8,28 ± 0,23	1,82 ± 0,24	8,14 ± 0,19	20,68 ±0,5	*3,28 ± 0,20*
Bp Rs3	0,77 ± 0,04; p = 0,11 vs. control	8,83 ± 0,09; p = 0,22 vs. control	2,064 ± 0,09; p = 0,24 vs. control	8,76 ± 0,51; p = 0,23 vs. control	21,23 ± 0,15; p = 0,38 vs. control	*4,95 ± 0,10; p = 0,006 vs. control*

TRM = Total reducing monosaccharides

TRD = Total reducing disaccharides

TRSP = Total reducing soluble polysaccharides

TRIP = Total reducing insoluble polysaccharides

TL = Total lipid

TSP = Total soluble protein

Previous studies have reported that rhizobacteria inoculation of soybean plant produced a wide range of effects in plant development (Lee, Gray et al., 2008). Rhizobacterial strains stimulate plant growth and enhance some physiological processes (Zhang, Dashti et al., 1997). BP Rs3 seed inoculation significantly increased plant height, number of leaves and foliar area, inducing in this way a plant growth promoting effect, without utilization of conventional fertilizers. It is well documented that *Bacillus* species are among the most isolated bacteria from plant tissues (Kobayashi and Palumbo, 2000). Other *B. pumilus* strains (isolated from the rhizosphere of *Alnus glutinosa*) had also a strong growth-promoting activity, but in greenhouse conditions (Probanza, Lucas et al., 1996), (Ramos, Lucas Garcia et al., 2003). These effects are mainly derived from morphological and physiological changes of the

BCUAS / CENTRAL UNIVERSITY LIBRARY

inoculated plant roots, leading to an enhancement of water and mineral uptake.

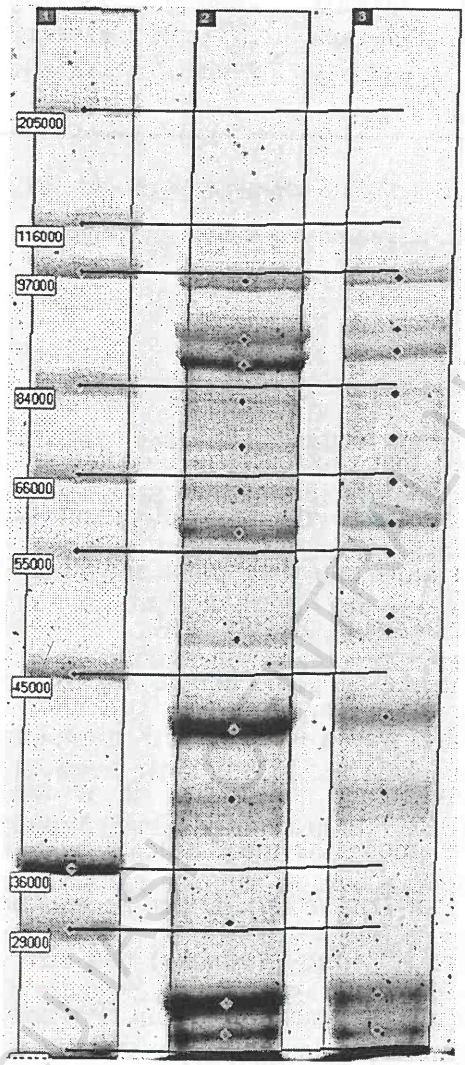


Fig. 17 – SDS-PAGE analysis of total soluble proteins from harvested beans. 1-Sigma wide range molecular weight marker; 2- plants inoculated with Bp Rs3; 3-non-treated control. Approx. 50 µg proteins were loaded per lane. Gel densitometry and molecular weight estimation were performed using ImageQuant TL software (GE Healthcare)

BCU LIBRARY UNIVERSITY LIBRARY

It is important to note that in field experiments the growth promotion provided by Bp Rs3 was apparently related to improved root development and enhanced nodulation, which resulted in better nutrient uptake capability and increased nitrogen supply. Enhanced nodulation is probably due to *B. pumilus* capabilities to produce gibberellins (Gutiérrez-Mañero, Ramos-Solano et al., 2001). This kind of effect was previously observed and the experiments demonstrate that *Bacillus* sp. strains enhance soybean nodulation and growth under low temperature stress (Bai, Zhou et al., 2003).

Although some studies involving plant growth promoting rhizobacteria inoculation revealed plants physiological changes (stimulation of carbohydrates biosynthesis) induced by the presence of rhizobacteria (Bashan, 1998), the results presented so far showed no significant differences between Bp Rs3 inoculated seeds and non-treated control regarding reducing soluble and insoluble carbohydrates content.

Some authors reported that rhizobacteria enhanced protein concentrations in plants (Sannazzaro, Ruiz et al., 2006). The above presented data showed that Bp Rs3 inoculation increased with 66% the total amount of seed soluble protein, probably due to stimulation of protein biosynthesis processes in soybean plants, providing in this way soybean seeds with higher nutritional value. Bp Rs3 treatment does not induce any qualitative changes of seed protein content (Stefan, Dunca et al., 2010).

Bp Rs3 rhizobacterial strain can be considered an eco-friendly alternative for reducing soil pollution caused by fertilizers usage. Bp Rs3

BCU IASI / CENTRAL UNIVERSITY LIBRARY

has a viable biotechnological potential to be used as biofertilizer, in the context of sustainable development and the respect for environment (Ianos, Peptenatu et al., 2009). Bp Rs3 strain is a spore-forming strain isolated from soybean rhizosphere which showed growth promoting effects on soybean plants. Its spore-forming characteristic makes Bp Rs3 more adaptable to commercial formulations in future inoculant production (Liu and Sinclair, 1993). However, to determine whether this strain is suitable for development into commercial biofertilizer, further experiments are necessary, especially to verify its plant growth promoting efficacy under a wider range of field conditions and with a greater number of soybean cultivars, and to evaluate relevant safety issues. Even if Bp Rs3 was isolated from soybean rhizosphere and it seems to represent a stable component of the bacterial population in soil, further studies are needed to investigate how repeated application of this culture would affect the balance with various indigenous soil bacteria.

1.2.3. Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean

The potential of two rhizobacterial strains to enhance physiological activities and yield of *P. coccineus* plants in low-input systems was investigated. The strains S4 (with P solubilization and siderophores production capabilities) and S7 (positive for IAA production) were tested as inoculants in order to confirm their PGP properties in field conditions. The field tests were carried out using the

BCU IASI CENTRAL UNIVERSITY LIBRARY

rhizobacterial strains in three different treatments: seed inoculation with S4, S7 and a combination of these strains (S4+S7). No data is available concerning the positive effects of PGPR strains on runner bean (Stefan, Munteanu et al., 2013).

Physiology of PGPR inoculated runner bean plants

The physiological measurements were performed following the main phenophases at 28 days after inoculation (DAI) – vegetative stage, 42 days DAI – early flowering stage, 59 days DAI – late flowering + fruit setting stage and 96 days DAI – fruit setting stage.

Changes in photosynthetic rates

PGPR inoculation of runner bean seeds resulted in higher photosynthetic rates in the case of all experimental samples compared with control at 28 and 42 DAI (Fig. 18). Initially photosynthetic activity increased during vegetative and early flowering stages but declined during fruit setting. According to Duncan's test there were significant increases of photosynthetic rates recorded for S7 ($p<0.004$) and S4+S7 ($p<0.003$) inoculated plants at 28 DAI and 42 DAI. No significant differences ($p>0.05$) could be observed for S4 treatment compared with non-inoculated runner bean plants. At 28 and 42 DAI, S4+S7 inoculated plants showed higher photosynthetic rates compared to plants inoculated with S4 and S7 strains. During late flowering + fruit setting stage (59

BCU IASI / CENTRAL UNIVERSITY LIBRARY

DAI) no PGPR positive influence on photosynthesis could be registered for inoculated runner bean plants. S7 strain showed the highest photosynthetic rate at 96 DAI, compared to S4 and S4+S7, but the recorded differences were non-significant.

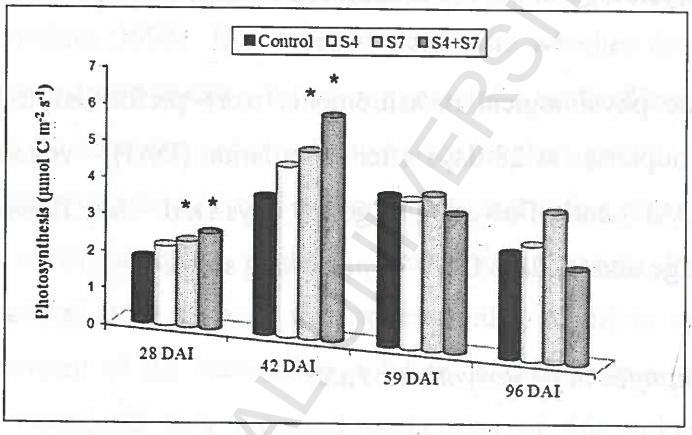


Fig. 18 – Effects of inoculation with PGPR strains S4, S7 and S4+S7 on photosynthesis in runner bean leaves. Values are mean ± S.E.M, (n = 9), * p<0.001 vs. Control

Transpiration

The results showed no significant differences among tested PGPR treatments concerning leaves transpiration during runner bean plants growth (Fig. 19). However, only during vegetative stage (28 DAI) co-inoculation with S4 and S7 increased significantly leaves transpiration (2.1 mmol H_2O m^{-2} s^{-1}), compared with control (p<0.004).

BOKU-IAS/CENTRAL UNIVERSITY LIBRARY

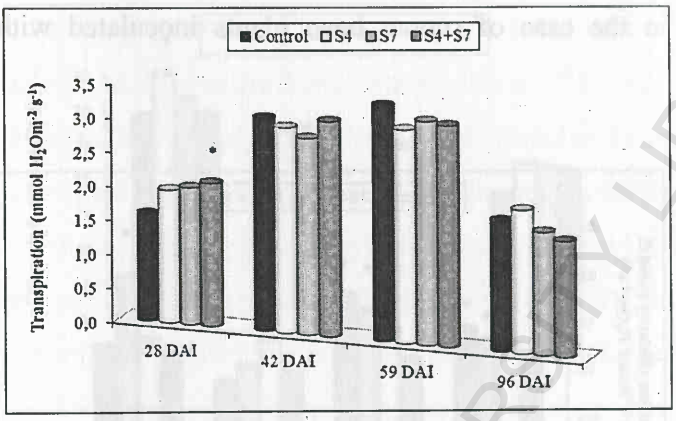


Fig. 19 – Effects of inoculation with PGPR strains S4, S7 and S4+S7 on transpiration in runner bean leaves. Values are mean ± S.E.M, (n = 9), * p<0.001 vs. Control

Water use efficiency

Water-use efficiency (WUE) increased in the presence of PGPR strains used to seed inoculation (Fig. 20). According to Duncan's test significant differences were observed only for S7 ($p<0.001$) and S4+S7 ($p<0.007$) treatments, compared with control. During vegetative and early flowering stage (28 and 42 DAI) all plants inoculated with a combination of the two tested strains showed higher values, compared to plants inoculated with a single rhizobacterial strain or without PGPR treatments. No significant differences could be observed at 59 DAI among tested rhizobacterial strains, compared with control. At the end of the experimental period (96 DAI) the highest water-use efficiency was

BCU IASI / CENTRAL UNIVERSITY LIBRARY

observed in the case of runner bean plants inoculated with S7 strain ($p<0.01$).

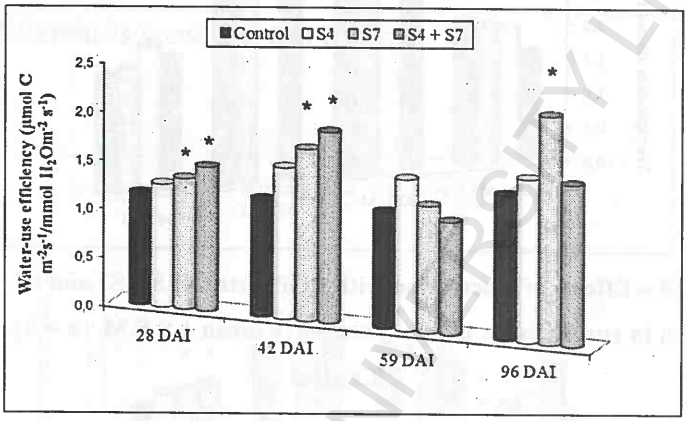


Fig. 20 – Effects of inoculation with PGPR strains S4, S7 and S4+S7 on water-use efficiency in runner bean leaves. Values are mean ± S.E.M, (n = 9), * $p<0.001$ vs. Control

Chlorophyll content

Chlorophyll content in leaves was slightly affected by different PGPR treatments (Fig. 21). However there was a significant chlorophyll content increase in leaves of runner bean plants inoculated with S4 strain ($p<0.01$), compared with control. This difference was recorded during vegetative stage (28 DAI). No significant differences could be observed for the other rhizobacterial strains used for seed inoculation.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

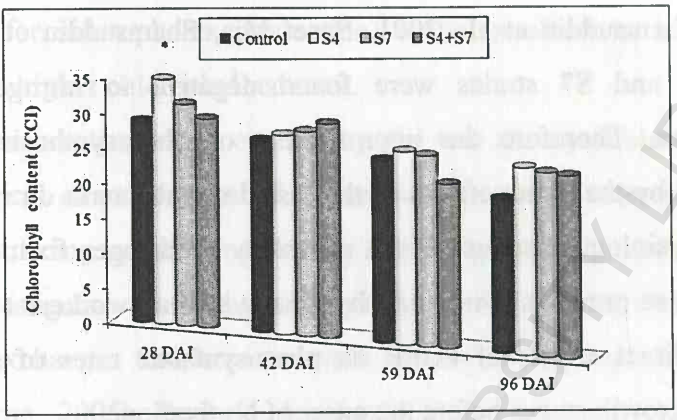


Fig. 21 – Effect of PGPR strains S4, S7 and S4+S7 on chlorophyll content in runner bean leaves. Values are mean ± S.E.M, (n = 10); * p<0.05 vs. Control

Seed inoculation with rhizobacteria exerted a positive effect on the physiological characteristics of the runner bean plants. This effect was significantly higher during vegetative and early flowering stages when a combination of two rhizobacteria (S4+S7) induced the highest photosynthetic activities, transpiration and water-use efficiency, compared with the other treatments and control. Photosynthesis was more sensitive to PGPR application than transpiration, similar to previous reports of Zhang et al. (1997). A direct positive effect of PGPR on photosynthesis was revealed, although the correlation between photosynthetic activities and PGPR influence is frequently presented in the literature only in an indirect way (Byrd and May, 2000, Oswald, Calvo Velez et al., 2010). The increase of physiological activity, namely photosynthesis is frequently associated with a higher N_2 fixation rate

BCU ASU CENTRAL UNIVERSITY LIBRARY

(Amir, Shamsuddin et al., 2001, Baset Mia, Shamsuddin et al., 2010). Both S4 and S7 strains were found negative to nitrogen fixation capabilities. Therefore the improvement of photosynthesis could be explained by the direct effects of the tested rhizobacteria on runner bean plants physiological status and not necessary by nitrogen fixation. Similar results were previously reported by Zhang and co-workers (1997) who showed direct effects of PGPR on photosynthetic rates of soybean at different growth stages before the onset of N_2 fixation.

Bacterial inoculations with S4 strain significantly increased leaves chlorophyll content during vegetative stage, the results agreeing with previous reports that presented significant increases of chlorophyll content in leaves of lettuce (Han and Lee, 2005) and basil (Heidari and Golpayegani, 2012) after inoculation with PGPR. Total chlorophyll content was correlated with higher iron acquisition (Katyal and Sharma, 1980) and may be used as a marker of iron availability to the plant system (Rasouli Sadaghiani, Barin et al., 2008). Also higher chlorophyll content could be related to higher activities of iron containing enzymes such as catalase and peroxidase, which are enhanced during the application of PGPR (Kavino, Harish et al., 2010). Catalase and peroxidase activities (97.37 micromoles H_2O_2/min/mg protein respectively 879.76 micromoles H_2O_2/min/µg protein) in the leaves of inoculated runner bean plants were significantly higher comparing with control (16.58 micromoles H_2O_2/min/mg protein - $p<0.009$, respectively 589.43 micromoles H_2O_2/min/µg protein - $p<0.02$). Therefore it is possible that S4 strain

BCU IAS / CENTRAL UNIVERSITY LIBRARY

(positive for siderophores production) to be involved in iron uptake, resulting a higher chlorophyll content.

The positive effect exerted by the two rhizobacterial strains on physiological characteristics of runner bean plants is more pronounced especially during vegetative and early flowering stages compared with the other investigated phenophases. This could be related with the root nutrient exchanges that become more intense during vegetative growth (Zamfirache, 2005). Because rhizobacteria mediates soil minerals and nutrients uptake (Sylvia, Fuhrmann et al., 1999) PGPR's influence is more visible during this stage. However, the data suggest that even during fruit setting period photosynthesis and WUE were increased by inoculation with S7 strain. This effect could be correlated with IAA producing capability of the rhizobacterial strain, although it is not clear how this strain enhanced photosynthesis during this period.

Effect of the rhizobacterial strains on runner bean plants antioxidant status

The activity of superoxide-dismutase and peroxidase in runner bean plants treated with PGPR was observed at three different times: 20, 42 and 59 DAI (Stefan, Munteanu et al., 2013). During vegetative stage, significantly higher SOD activity was obtained in the leaves of S7 inoculated plants compared with control. Also, S4 and S4+S7 treatments induced higher SOD activities comparing with the control but the recorded differences where not significant (Fig. 22). Co-inoculation

BCU "AS" CENTRAL UNIVERSITY LIBRARY

induced the highest peroxidase activity at 20 DAI (Fig. 23). Also at the same time, plants treated with S7 strain showed a milder, but significant increase of peroxidase activity, compared with the control. At 42 DAI the highest enzymatic activity was recorded for S4 treated plants. No significant differences were recorded for the other treatments versus control at 42 DAI. The PGPR inoculation of runner bean plants did not influence peroxidase activity at 59 DAI.

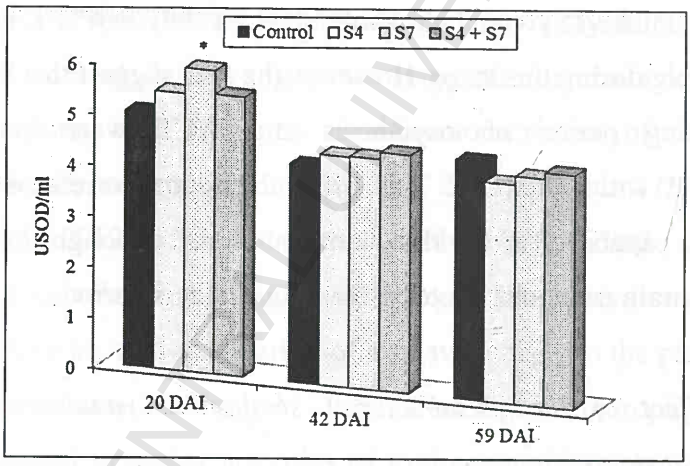


Fig. 22 - Effect of PGPR strains S4, S7 and S4+S7 on superoxide-dismutase activity in runner bean leaves. Values are mean ± S.E.M, (n = 3). * p<0.005 vs. control. DAI = days after inoculation

The activity of investigated antioxidant enzymes was increased in the presence of PGPR strains (Stefan, Munteanu et al., 2013). SOD activity was significantly higher in the leaves of S7 inoculated plants at 20 DAI, compared with the control. Also, an enhancement of peroxidase

BCU IASI / CENTRAL UNIVERSITY LIBRARY

activity at 20 DAI was recorded in the case of S4 and S4 + S7 treatments and at 42 DAI for S4 strains inoculated plants.

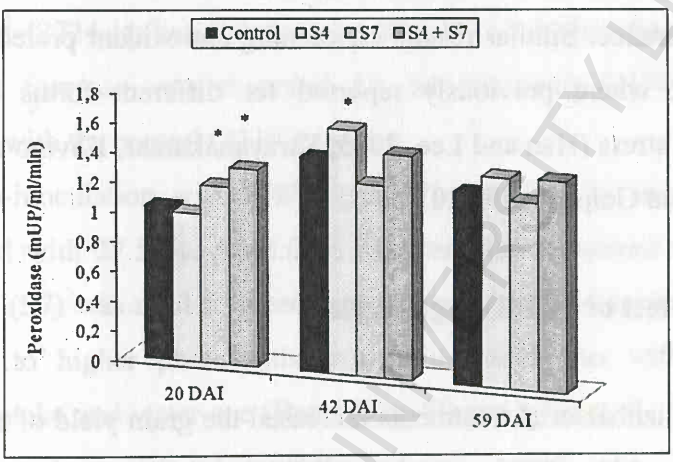


Fig. 23 - Effect of PGPR strains S4, S7 and S4+S7 on peroxidase activity in runner bean leaves. Values are mean ± S.E.M, (n = 3). *p<0.005 vs. control. DAI = days after inoculation

Normally, these antioxidant enzymes tend to detoxify the toxic H_2O_2 accumulated during intense photosynthetic activities as it was recorded in the case of runner bean plants treated with S7 strain at 20 DAI and for S4 inoculated plants at 42 DAI. Efficient removal of reactive oxygen intermediates from chloroplasts is critical, since H_2O_2 concentrations as low as 10 μM can inhibit photosynthesis by 50% (Kaiser, 1979). Also it is clearly stated that the over expression of antioxidant enzymes such as SOD in chloroplasts provides increased protection from oxidative stress (Allen, 1995). Therefore it may be

BCU IASI CENTRAL UNIVERSITY LIBRARY

considered that S4 and S7 strains can prevent oxidative stress by increasing SOD and peroxidase activities during periods with intense photosynthesis; this elevated activity could be correlated with increased stress tolerance. Similar results concerning antioxidant protective effects of PGPR where previously reported for different forms of induced oxidative stress (Han and Lee, 2005, Saravanakumar, Kavino et al., 2011, Heidari and Golpayegani, 2012).

Effect of PGPR on grain yield

Rhizobacterial treatments increased the grain yield of runner bean cultivated under organic growing conditions, the results being presented in Table 4.

Table 4 – Effect of PGPR strains S4, S7 and S4+S7 on runner bean yield. Values are mean (n = 10)

Treatments	Yield (kg/ha)	Differences vs. Control (kg)	Significance of the differences vs. control
S4	2194.9	76.5	ns
S7	2437.3	318.8	*
S4+S7	2724.0	605.6	***
Control	2118.4	0	ns

LSD 5%=235.9 kg, LSD 1%=357.3 kg, LSD 0.1%=573.9 kg

* - significant positive differences

*** - very significant positive differences

ns – no significant differences

BCUIASI / CENTRAL UNIVERSITY LIBRARY

Significant grain yield increase was obtained with S4+S7 ($p < 0.008$) and S7 ($p < 0.01$) treatments by comparison with the control. Plants inoculated with a combination of S4 and S7 strains produced the highest grain yield (2724 kg/ha) followed by S7 (2437.3 kg/ha) treatment. No significant increase was recorded for S4 treatment (2194.9 kg/ha), compared with the control (2118.4 kg/ha).

Co-inoculation with PGPR (S4+S7) significantly increased the grain yield with 27.58%. A 15.03% increase was registered when only one strain (S7) was used for seed inoculation. The yield increase can be attributed to higher photosynthetic activities and also with efficient nutrient uptake and water use (Baset Mia, Shamsuddin et al., 2010). Co-inoculation increased photosynthesis and water use efficiency, maintaining in this way the physiological activities at a higher level which is essential to increase the yield. The results are in accordance with Peng et al. (2002), which showed that rice inoculated with *Rhizobium* had greater rates of photosynthesis and therefore greater dry matter production and yield (Peng, Jatish et al., 2002). Also Deka Boruah and co-workers demonstrated a similar connection among inoculation with *Pseudomonas* and net assimilation rate/growth and yield of french bean (Deka Boruah, Rabha et al., 2003).

Many studies and surveys reported increased yield and some other elements due to PGPR inoculation (Singh, Pandey et al., 2011). It is well documented that PGPR can increase crop growth by a combination of mechanisms, which include phytohormone production, increasing the availability of soil nutrients, nitrogen fixation (Bashan, 1998, Cocking,

BCU AST CENTRAL UNIVERSITY LIBRARY

2003, Vessey, 2003). Since both S4 and S7 strains were found negative to nitrogen fixation trait it may be presumed that the yield enhancement effects could be explained by IAA producing and P solubilizing capacities of the tested strains.

Protein, carbohydrate and lipid bean content

Plants inoculated with PGPR showed higher seed protein-content compared to control plants (Table 5) (Stefan, Munteanu et al., 2013).

Table 5 – Total soluble protein, reducing carbohydrates and lipid content of harvested beans

Treatment	Total soluble protein (mg/g harvested seeds)	Total reducing carbohydrates (mg/g harvested seeds)	Total lipid (mg/g harvested seeds)
Control	123.06 ± 5,00	4.20 ± 0,86	38.17 ± 4,53
S4	139.83 ± 6,29*	6.27 ± 0,86*	36.51 ± 4,84
S7	143.05 ± 4,72*	5.19 ± 0,79*	36.93 ± 2,86
S4 + S7	128.10 ± 3,49	5.23 ± 0,63*	38.48 ± 5,24

- values are means ± standard deviation of three separate determinations.

* - significant differences vs. control ($p<0.05$)

Thus inoculation with S7 strain induced a 16.24% increase of runner bean soluble protein yield, followed by S4 treatment (139.83 mg/g harvested seeds). Co-inoculation determined also an increase of seed protein yield versus control, but the recorded differences where not

BCU IASI / CENTRAL UNIVERSITY LIBRARY

significant. Similar pattern was also showed for total reducing carbohydrates seed content (Table 5). In this case both inoculation and co-inoculation with PGPR strains induced an increase of carbohydrates bean content. An increase of 49.28% was achieved by inoculation with S4 strain, followed by S4 + S7 (24.52%) and S7 (23.57%) treatments. PGPR inoculation of runner bean plants did not induced any significant differences regarding the total lipid content of harvested beans compared with non-inoculated plants (Table 5).

Inoculation and co-inoculation of runner bean plants with S4 and S7 strains influenced the nutritional value of the harvested grains. Thus, PGPR increased seed total soluble protein content up to 16.24%. Also both inoculation and co-inoculation enhanced the total reducing carbohydrates content of the beans up to 49.28%, in the case of S4 treatment. Improvement of grain protein yield where also reported by (Dashti, Zhang et al., 1997) in the case of soybean plants under short season conditions. The protein content enhancement is related to a higher relative increases in nitrogen fixation due to PGPR inoculation. The two PGPR strains used for seed inoculation were found negative for nitrogen fixation capabilities. Therefore the increase of grains total soluble protein content could be related to enhancement of physiological activities of runner bean plants and subsequent to growth.

Similar results were previously reported for soybean plants grown in organic conditions (Stefan, Dunca et al., 2010). In this case, inoculation with *Bacillus pumilus* Rs3 strain increased with 66% the total amount of seed soluble protein, probably due to stimulation of protein biosynthesis

BCU IASI / CENTRAL UNIVERSITY LIBRARY

processes in soybean plants. Considering that beans provide dietary proteins that play an essential role in human nutrition by complementing other foods (Broughton, Hernández et al., 2003) S4 and S7 strains could be used in future agricultural biotechnology in order to improve the nutritive value of runner bean plants (Stefan, Munteanu et al., 2013).

In conclusion PGPR can increase photosynthesis, WUE, chlorophyll content and yield of runner bean plants. The main mechanisms involved are mobilization of insoluble nutrients and subsequent enhancement of uptake and production of siderophores/plant growth regulators that stimulate plant growth (Zhang, Dashti et al., 1997). One of the tested strains namely S4 was found positive for phosphate solubilization and siderophore production. In a phosphate solubilization broth assay S4 decreased the culture medium pH from 7.2 to 3.7 which clearly indicates the production of organic acid or phosphatase (Chaiharn and Lumyong, 2011). It has been shown that low pH values favor the production of acid phosphatase and phytase activities by soil microorganisms (Gargova, Roshkova et al., 1997). Considering that P availability is a limiting step in plant nutrition (Goldstein, 1986) and microorganisms that decrease the medium pH during growth are efficient P-solubilizers (Khan, Bhuiyan et al., 2006), S4 plays an important contribution as a phosphate-solubilizing bacteria to plant nutrition and therefore to the increasing of photosynthetic activities and yield. Similar results are presented in the literature, being a known fact that the use of PSB as inoculants improves plant growth performance and

BOU IASI CENTRAL UNIVERSITY LIBRARY

simultaneously increases P uptake by the plant and crop yield (Richardson, 1994, Rodríguez and Fraga, 1999, Fernández, Zalba et al., 2007).

The other strain – S7 was selected for the study as the best IAA producing rhizobacteria (4.86 µg IAA/ml). S7 produced IAA in the presence of L-tryptophan (0.204 g/l) as precursor. This data suggest that the strain is dependent of this precursor and probably synthesized IAA through tryptophan pathways (Chaiharn and Lumyong, 2011). L-tryptophan is excreted by the plant roots under natural conditions (Ahmad, Ahmad et al., 2005) and can be used by rhizobacteria to synthesize IAA related to enhance root proliferation (Spaepen, Vanderleyden et al., 2007). This increase root development and further plant mineral uptake, stimulating indirectly plant growth (Lambrecht, Okon et al., 2000).

The results indicate that co-inoculation produced a more pronounced influence while inoculation alone showed a lower effect. In addition, a combination of two strains (S4+S7) increased physiological activities while one strain alone (S4) increased chlorophyll content. This data can suggest the existence of a synergistic effect between rhizobacterial inoculated strains. The hypothesis of synergic actions is also reported by Rojas and co-workers and their conclusion is that in normal conditions it is not uncommon to see the promoting plant growth effects of rhizobacteria *via* a synergism of the rhizospheric microorganism (Rojas, Holguin et al., 2001). Also, PGPR co-existing in the rhizosphere that have single modes of action may act synergistically to stimulate the growth of the host plant (Vessey, 2003). Bai et al. (2003) reported that co-inoculation of *Bradyrhizobium japonicum* with *Bacillus*

BCU IASI / CENTRAL UNIVERSITY LIBRARY

thuringiensis NEB17 provided the largest and most consistent increases in nodule number, nodule weight, shoot weight, root weight, total biomass and grain yield (Bai, Zhou et al., 2003). That's why in agricultural inoculation practices, mixing of two or more microbial species often has a more positive effect on plant growth than the use of a single bacterium (Belimov, Kojemiakov et al., 1995).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Chapter 2.
ANTIMICROBIAL APPLICATIONS OF DIFFERENT NATURAL AND ARTIFICIAL COMPOUNDS

Marius ȘTEFAN, Marius MIHĂȘAN

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Introduction

Despite the impressive scientific progress in vaccination and chemotherapy, infectious diseases remain a serious health issue. Following the massive and inappropriate use of antibiotics, bacteria have developed various mechanism of resistance; consequently, infectious diseases remain one of the leading causes of morbidity worldwide. Therefore the pharmaceutical companies and the researchers are in a continuous quest for new antibacterial agents (Rai, Yadav et al., 2009). In this direction, some studies were focused on the investigation of the antibacterial effects of different natural (plant extracts) and artificial (noble metal nanoparticles) compounds.

2.1. COMPOSITION AND ANTIBACTERIAL ACTIVITY OF BASIL ESSENTIAL OILS

In recent years, the essential oils and herbal extracts have attracted a great deal of scientific interest due to their potential as a source of natural antioxidants and biologically active compounds. Moreover, it must be noted that different plant products or extracts in combinations with chemosynthetics or antibiotics are able to exhibit synergy effects, these co-medications being of beneficial interest in a future therapy (Wagner and Ulrich-Merzenich, 2009).

Basil is one of the oldest spices belonging to the *Ocimum* genus and to the *Lamiaceae* (*Labiatae*) family, widely distributed in tropical and

BCU IASI / CENTRAL UNIVERSITY LIBRARY

warm temperature regions. In Romania it is frequently used in cuisine (food flavouring and preservation), alternative medicine, for pharmaceutical products and in cosmetics industry. It has been reported that the volatile oil of this plant contains mostly phenols, particularly thymol - compounds probably responsible for its reported antimicrobial action (Dambolena, Zunino et al., 2010).

O. basilicum (sweet basil), *O. gratissimum* (clove basil or African basil) and *O. tenuiflorum* (syn. *O. sanctum* - holy basil) are the most used *Ocimum* species as medicinal and aromatic plants. Because of the importance of *Ocimum* species and of the great variability in chemical composition, the essential oils constituted the object of many studies, but very little is known about basil species growing in Romania (Benedec, Oniga et al., 2009).

Antimicrobial activity of essential oils from some of the *Ocimum* species (especially *O. basilicum* and *O. gratissimum*) has been previously studied, but as the chemical composition of oil varies according to the population or chemotype analyzed, different antimicrobial activity was observed depending on the origin of the oils (Ngassoum, Essia-Ngang et al., 2003). Therefore, it is necessary to investigate the morphology of the volatile oil-producing glandular hairs in relation with chemical composition and antimicrobial activity of the volatile oils (Stefan, Zamfirache et al., 2013).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Chemical composition of the essential oils

The composition of the volatile fractions obtained from plants in blooming stage belonging to the three *Ocimum* investigated species is presented in Table 6.

Table 6 – The composition of the volatile fractions obtained from the investigated *Ocimum* species

No.	Chemical compound	*O. gratissimum*	*O. tenuiflorum*	*O. basilicum* var. *Genovese*
		RA (%)	RA (%)	RA (%)
1	1,8 cineole	2.51	0.93	1.5
2	β-ocimene	0.59	2.63	3.54
3	linalool	74.22	38.6	65.38
4	α-terpineol	0.34	-	0.45
5	eugenol	5.8	11.82	5.26
6	β-elemen	0.66	1.44	0.82
7	α-bergamotene	3.6	0.94	1.55
8	α-guaiene	0.68	1.89	0.96
9	germacrene D	3.22	6.08	1.46
10	δ-guaiene	1.52	4.41	2.27
11	γ-cadinene	0.86	1.86	1.1
12	cubenol	0.28	0.98	0.84
13	tau-cadinol	3.47	10.2	8.18
14	camphor	0.92	1.85	2.13
15	bornyl acetate	0.19	0.35	0.74

BCU IASI / CENTRAL UNIVERSITY LIBRARY

No.	Chemical compound	*O. gratissimum*	*O. tenuiflorum*	*O. basilicum* var. *Genovese*
		RA (%)	RA (%)	RA (%)
16	β-caryophyllene	0.44	2.13	1.17
17	α-caryophyllene	0.47	1.64	0.54
18	terpinolene	-	0.25	0.19
19	mentone	-	1.01	-
20	mentofuran	-	0.46	-
21	menthol	-	2.28	-
22	δ-terpineol	-	0.35	-
23	pulegone	-	0.52	-
24	neomenthyl-acetate	-	0.22	-
25	γ-muurolene	-	0.99	0.15
26	elixene	-	2.32	0.28
27	trans nerolidol	-	0.48	0.4
28	δ-cadinol	-	0.81	0.58
29	borneol	-	-	0.17
30	nerol	-	-	0,25
	TOTAL	99.77	97.44	99.91

RA = Relative area percentage (peak area relative to the total peak area percentage).

Thirty compounds representing about 97.44 – 99.91% of the total oil were identified. The highest number of different compounds was identified in the *O. tenuiflorum* oil (27 compounds) and the lowest in *O. gratissimum* oil (17 compounds). Linalool was identified as the major component of all investigated basil oils, representing 74.22% in *O. gratissimum* oil, 65.38% in *O. basilicum* var. *Genovese* oil and 38.60% in *O. tenuiflorum* oil. Other major compounds found were eugenol and tau-

BCU MASINO CENTRAL UNIVERSITY LIBRARY

cadinol, followed by α-bergamotene and 1,8-cineole (in *O. gratissimum* oil), germacrene D (in *O. tenuiflorum* and *O. gratissimum* oil) and β-ocimene (in *O. basilicum* var. *Genovese* oil).

The chemical composition of the essential oils belonging to the investigated basil taxa is varied. Considering that all of the plants were cultivated under the same conditions the impact of ecological factors on essential oil composition variability has been excluded. Also the plants were harvested in the same phenophase, therefore the differences in essential oils composition are determined only by genetic factors.

According to Marotti and co-workers (Marotti, Piccaglia et al., 1996) all the tested essential oils are of type 1 with linalool as the main component. The biosynthesis site of linalool in *Lamiaceae* species is in peltate glandular hairs with multicellular glands (Schie, Haring et al., 2007). The electron microscopy studies revealed the presence of a high density of peltate hairs on lower epidermis of the petals and sepals in *O. basilicum* var. *Genovese* and *O. gratisimum*. Therefore the high amount of linalool found in *O. gratissimum* and *O. basilicum* var. *Genovese* oils (74.22% respectively 65.38%) could be correlated with larger and more numerous peltate hairs from the calyx and corolla elements. Also it must be taken into consideration that the harvesting was done in anthesis stage when the proportion of flowers in plant material mass was important and the leaves rich their final dimensions.

The results are in a good agreement to those of Zheljazkov et al. who reported that the major oil constituent of *Ocimum basilicum* L and. *O. tenuiflorum* L. from Mississippi (USA) was linalool (30 - 40%) and

BCU IASI / CENTRAL UNIVERSITY LIBRARY

eugenol (8 - 30%) (Zheljazkov, Cantrell et al., 2007). Also Hussain et al. showed that the most abundant component of essential oils extracted from *O. basilicum* indigenous to Pakistan was linalool (56.7 - 60.6%) (Hussain, Anwar et al., 2008). According to Carović-Stanko and co-workers linalool was the main compound identified in the hydro-distilled *O. basilicum* var. *Genovese* essential oil from Croatia (66.40%) (Carović-Stanko, Orlić et al., 2010). In other *O. basilicum* chemotypes linalool is absent and in *O. tenuiflorum* this compound is present in small amounts (Kothari, Bhattacharya et al., 2004).

The comparison of the results with the data reported in literature showed significant differences concerning oil composition, which can be attributed to the local climatological factors, genetic differences between populations, different chemotypes and plants nutritional status or to the development stage of the analyzed plants. Variation in chemical pattern of *O. basilicum* essential oil has been also reported by (Dambolena, Zunino et al., 2010) who found that linalool and eugenol varied widely with harvest time and varieties.

Antimicrobial activity

The diameters of growth inhibition zones measured by well diffusion method are presented in Table 7. The results suggest that all essential oils showed antibacterial activity against both tested organisms, depending on their concentration, with inhibition zones ranging from 1 to 15.7 mm. *O. basilicum* var. *Genovese* oil produced the strongest

BU/IASI/CENTRAL UNIVERSITY LIBRARY

antibacterial effect on *S. aureus* and *E. coli*. Moderate effects were registered in the case of *O. gratisimum* and *O. tenuiflorum* oils for both tested organisms. There was no inhibition of growth observed when DMSO was used as control.

Table 7 – Antimicrobial activity of *Ocimum* essential oils against *Staphylococcus aureus* and *Escherichia coli* using well diffusion assay. The diameter of inhibition zone includes the well (5 mm)

Essential oil sample	Diameter of inhibition zone (mm) Bacterial strain					
	S. aureus			*E. coli*		
Concentration (mg/ml)	45	90	180	45	90	180
O. gratisimum	8.7 ± 0.5	8.0 ± 0.2	11.3 ± 0.6	10.6 ± 0.5	11.0 ± 1.0	11.0 ± 0.1
O. tenuiflorum	8.0 ± 0.2	9.0 ± 0.2	10.0 ± 0.1	8.6 ± 0.5	9.7 ± 0.5	10.7 ± 0.5
O. basilicum var. *Genovese*	9.3 ± 1.5	12.7 ± 1.7	15.7 ± 1.2	8.0 ± 1.0	11.0 ± 1.0	12.0 ± 1.0
Gentamicin (10 µg/disc)		23.0 ± 1.0			19.7 ± 0.5	

Values are mean inhibition zone (mm) ± S.D. of three replicates;

The bacteriostatic and bactericidal concentrations of essential oils obtained by colorimetric assay followed by plating on MHA are presented in Table 8.

In the case of *S. aureus* the lowest MIC (minimum inhibitory concentration – 0.364 mg/ml, w/v) was determined by *O. tenuiflorum* and *O. basilicum* var. *Genovese* oils. *E. coli* was more susceptible to *O.*

BCU IASI / CENTRAL UNIVERSITY LIBRARY

gratisimum and *O. basilicum* var. *Genovese* essential oils (MIC = 0.364 mg/ml, w/v). The minimum concentration of essential oil necessary to kill an organism (MBC) should be equal to or greater than the MIC for that microbe (Mann and Markham, 1998). Both bacterial strains presented MBCs which were two or even three times higher comparing with MIC.

Table 8 – Minimum inhibitory concentrations and minimum bactericidal concentrations of *Ocimum* essential oils (mg/ml, w/v) against *Staphylococcus aureus* and *Escherichia coli*

Sample	Bacterial strain			
	S. aureus		*E. coli*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
O. gratisimum	0.728	1.456	0.364	1.456
O. tenuiflorum	0.364	1.456	0.728	1.456
O. basilicum var. *Genovese*	0.364	0.728	0.364	0.728

MIC = minimum inhibitory concentration

MBC = minimum bactericidal concentration

The oils presented good antibacterial activity against both *E. coli* and *S. aureus*, the MICs values being in accordance with the data presented in the literature (Bozin, Mimica-Dukic et al., 2006). The growth of *E. coli* was inhibited especially by *O. gratisimum* and *O. basilicum* var. *Genovese* oils, probably due to its high linalool content (74.22 and 65.38%). The inhibition zones (10.7 ± 0.5 mm) and the MIC value (0.728 mg/ml, w/v) obtained in the case of *O. tenuiflorum* oil suggest a less

BCU CAS CENTRAL UNIVERSITY LIBRARY

pronounced antimicrobial effect that can be correlated with a lower linalool content (38.60%).

O. basilicum var. *Genovese* and *O. tenuiflorum* oils exhibited the strongest antibacterial effect against *S. aureus* (MIC value - 0.364 mg/ml, w/v) even if their linalool content was lower compared with *O. gratissimum* oil (65.3%, respectively 38.60%). In this case, the increased antibacterial properties can be related with higher content of eugenol (5.26%, respectively 11.82%). One possible reason for this result could be the synergistic action of the constituents in the oils (Suppakul, Miltz et al., 2003). Prasad and co-workers studied the antimicrobial activity of essential oils of *O. basilicum* rich in linalool, methyl chavicol, and methyl cinnamate against eleven Gram-positive and seven Gram-negative bacteria and they found that these oils were more effective against Gram-positive than against Gram-negative bacteria (Prasad, Kuman et al., 1986). Also Lahariya and Rao studied the antimicrobial effectiveness of the essential oil of *O. basilicum* tested *in vitro* against 10 different microorganisms and showed that this essential oil had no activity against *Staphylococcus* (Lahariya and Rao, 1979). In the case of basil oils both *E. coli* and *S. aureus* were inhibited by each of the tested essential oils.

The antimicrobial activity of the three basil species essential oils could be explained by the significant amount of linalool, an oxygenated monoterpenoid. This compound is previously known for its antimicrobial activity (Kim, Marshall et al., 1995). The mechanism involved is related to its high water solubility and therefore to its ability to penetrate the cell walls of a bacterium (Knobloch, Pauli et al., 1989). Thus, the

BCU IASI CENTRAL UNIVERSITY LIBRARY

antimicrobial activity of essential oils is due to their solubility in the phospholipid bilayer of cell membranes. Also linalool is active against microorganisms potentially acting as either a protein denaturing agent or as a solvent dehydrating agent.

Knobloch et al. demonstrated that linalool, with its high water solubility, had a significant antimicrobial activity as compared to eugenol (Knobloch, Pauli et al., 1989). Nevertheless the antibacterial activity of the basil examined essential oils can be related to the content of other identified compounds, such as eugenol (Dorman and Deans, 2000). Although linalool presents important antioxidant and antimicrobial effects (Queiroga, Teixeira Duarte et al., 2007), it must be noted that the antimicrobial effect of an essential oil depends on all its chemical components.

The essential oils from the fresh herbs (especially from *Ocimum basilicum* L. var. *Genovese*) could be used as antimicrobials for food preservation and in pharmaceutical or cosmetic industries. However, further evaluations are needed in order to determine if these essential oils have a negative impact on human normal microbiota.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

2.2. ANTIBACTERIAL ACTIVITIES OF SOME METHANOLIC PLANT EXTRACTS AGAINST MULTIDRUG-RESISTANT BACTERIA

One of the main medical problems is the widespread emergence of acquired bacterial resistance to antibiotics in such a way that the world is facing today a serious threat to global public health in the form of not only epidemics, but also pandemics of antibiotic resistance (Chanda, Baravalia. Y. et al., 2010). Several mechanisms have been accounted for, but active efflux plays an important role in this phenomenon (Pages, Lavigne et al., 2009). The accumulation of different antibiotic resistance mechanisms within the same strains has led to the appearance of the so called superbugs, or multi-drug resistant bacteria (Chopra, 2000). Due to this antibiotics resistance problem, attention is now being shifted towards biologically active components isolated from plant species commonly used as herbal medicine, as they may offer a new source of antibacterial, antifungal and antiviral activities (Maiyo, Ngure et al., 2010). The potential antimicrobial properties of plants are related to their ability to synthesize several secondary metabolites of relatively complex structures possessing antimicrobial activities (Matasyoh, Maiyo et al., 2009). In addition, promising new concepts such as the efflux pump inhibitors (Newman and Cragg, 2007), and synergy between antibiotics and phytochemicals are now beeing explored.

BCU IASI CENTRAL UNIVERSITY LIBRARY

2.2.1. Antibacterial activities of some vegetables extracts

Among medicinal plants, vegetables associated to non or less-toxic effects have been shown to possess many medicinal properties including antibacterial effects (Huie, 2002). Therefore, some studies were designed to investigate the antibacterial effects of Cameroonian vegetables namely *Amarantus hybridus* Linn (*Amarantaceae*), *Vernonia hymenolepis* (H.F.) Hook., *Lactuca sativa* Linn. and *Lactuca capensis* Thumb. (*Asteraceae*), *Manihot esculenta* Crantz (*Euphorbiaceae*), *Phaseolus vulgaris* Linn (*Fabaceae*), *Cucurbita pepo* Linn and *Sechium edule* (Jacq) Sw. (*Cucurbitaceae*), *Solanum nigrum* Linn. and *Capsicum frutescens* L. (*Solanaceae*) against multi drug resistant (MDR) bacteria expressing active efflux pumps (Noumedem, Ștefan et al, 2013).

Chemical composition of the vegetable extracts

The results of the qualitative analysis showed that each of the studied plant extract contains at least two classes of secondary metabolites such as alkaloids, anthocyanins, anthraquinones, flavonoids, phenols, saponins, steroids, tannins and triterpenes (Table 9).

Only the extract from *A. hybridus* contains anthocyanins, while triterpenes were found both in this extract as well as that of *C. frutescens*. The extract from *C. frutescens* as well as those from *S. edule* and *M. esculenta* contained the highest number of classes of the studied

BCU IASI / CENTRAL UNIVERSITY LIBRARY

secondary metabolites (five). Alkaloids and phenols were present in all vegetable extracts except that of *A. hybridus*.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Table 9 – Extraction yields and phytochemical composition of the plant extracts

Scientific names	Part used	Yield (%)	alkaloids	phenols	tannins	terpènes	stéroids	flavonoids	anthraquinones	anthocyanins	saponins
Amarantus hybridus	leaves	7.9	-	-	-	+	-	+	-	+	-
Vernonia hymenolepis	leaves	9.40	+	+	-	-	-	+	-	-	-
Lactuca sativa	leaves	7.14	+	+	-	-	-	+	-	-	-
Lactuca capensis	leaves	7.14	+	+	+	-	+	-	-	-	-
Sechium edule	leaves	3.76	+	+	-	+	+	+	-	-	+
Manihot esculinta	leaves	07.46	+	+	+	+	+	+	+	-	+
Phaseolus vulgaris	cloves	17.81	+	+	-	-	+	+	-	-	-
Cucurbita pepo	leaves	12.68	+	+	-	-	+	+	-	-	-
Solanum nigrum	leaves	11.84	+	+	-	-	+	+	-	-	-
Capsicum frutescens	fruits	16.24	+	+	-	+	-	+	+	-	-

(+): Present; (-): Absent;
The yield was calculated as the ratio of the mass of the obtained methanol extract/mass of the plant powder

BCU IASH/CENTRAL UNIVERSITY LIBRARY

Antibacterial activity of the vegetable extracts

The data summarized in Table 10 show the antibacterial activities of the tested extracts on a panel of twenty-nine Gram-negative bacteria. All extracts were active on at least twelve bacterial strains with MIC ≤ 1024 µg/ml. The extract of *C. frutescens* showed inhibitory activities against 16 (55.17%) of the 29 tested bacteria whilst that of *P. vulgaris* inhibited the growth of 12/29 (41.38%) pathogens (narrowest spectrum). None of these two extracts showed any antibacterial activity against *Pseudomonas* species, but were active against at least one bacterial strain of other studied genus. Extracts from *L. sativa*, *S. edule*, *C. pepo* and *S. nigrum* displayed the largest spectra of activity, their inhibitory effects being observed on all the 29 Gram negative bacteria (100% of activity). The extracts from *A. hybridus*, *V. hymenolepis*, *L. sativa*, *L. carpensis* and *M. esculenta* also exhibited large spectrum of activity as they were active on 28/29 tested bacteria. The top eight active extracts, with large spectra of activity, showed MIC values generally ranging from 128 to 512 µg/ml. These MIC values were in some of the cases better than those of choramphenicol (Table 10). This was the case with the extract from *V. hymenolepis* (MIC of 128 µg/ml) against *E. aerogenes* EA27. The extracts from *A. hybridus*, *S. edule* and *C. pepo* as well as those from *L. capensis* and *M. esculenta* were more active than chloramphenicol on at least one of the tested MDR bacteria. The activity of chloramphenicol increased in the presence of PAβN (an efflux pump inhibitor) in the majority of the tested bacteria (Table 10). The best activity was obtained

BCU ASI CENTRAL UNIVERSITY LIBRARY

with the extract from *A. hybridus* with the lowest MIC value of 128 µg/ml observed against 7/29 (25%) tested bacteria. The extracts from *P. vulgaris* and *C. frutescens* did not show any MBC value at up to 1024 µg/ml. In the case of the other eight vegetable extracts, the MBC results showed values equal to or below 1024 µg/ml in many cases. The extract from *C. pepo* leaves showed the best MBC spectrum with the values below to 1024 µg/ml recorded on 58.62% (17/29) of the studied microorganisms, followed by those from *M. esculenta* leaves on 51.72% (15/29), *A. hybridus*, *V. hymenolepis* and *L. capensis* extracts on 44.83% (13/29) and *L. sativa* on 31.03% (9/29) (Table 11). The same table also shows that *M. esculenta* exhibited MBC values against all the strains of *E. aerogenes* and that, in general, the extracts presented values which were not 4-fold greater than the corresponding MICs.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Table 10 – Susceptibility of bacteria to plant extracts - MICs of methanol extracts *vs.* chloramphenicol

Bacterial strains	MIC (µg/ml) of the plant extracts										
	A. hybridus	*V. hymenolepis*	*L. sativa*	*L. capensis*	*S. edule*	*M. esculenta*	*P. vulgaris*	*C. pepo*	*S. nigrum*	*C. frutescens*	Chloramphenicol
E. coli											
ATCC8739	256	1024	512	512	256	256	1024	512	512	512	4
ATCC10536	128	256	128	256	128	256	-	256	128	-	4
W3110	256	512	256	256	256	512	-	128	256	-	8 (<2)
MC4100	512	1024	512	1024	256	512	1024	256	512	1024	16 (<2)
AG100A	256	512	512	512	256	512	-	512	512	1024	< 2 (<2)
AG100Atet	256	512	512	512	256	512	-	512	512	1024	64(< 2)
AG102	1024	128	1024	512	512	128	-	-	256	512	64 (<2)
AG100	128	1024	128	512	512	512	-	256	128	-	8 (< 2)
E. aerogenes											
ATCC13048	128	1024	256	256	256	256	1024	256	256	-	8
EA294	512	512	512	512	512	1024	-	512	512	1024	16
CM64	128	128	256	256	128	256	1024	512	256	512	256 (8)
EA3	256	256	128	128	256	128	1024	128	128	-	256(128)
EA298	256	512	256	256	256	256	1024	128	256	1024	64(< 2)
EA27	512	128	256	-	512	512	-	512	256	512	≥ 256(< 2)
EA289	-	512	1024	256	512	256	1024	128	1024	256	≥ 256 (64)
K. pneumoniae											
ATCC11296	256	512	256	512	512	512	512	256	256	-	8
KP55	256	512	256	512	256	512	1024	256	256	256	32 (4)

BCU IASI CENTRAL UNIVERSITY LIBRARY

Bacterial strains	MIC (µg/ml) of the plant extracts										
	A. hybridus	*V. hymenolepis*	*L. sativa*	*L. capensis*	*S. edule*	*M. esculenta*	*P. vulgaris*	*C. pepo*	*S. nigrum*	*C. frutescens*	Chloramphenicol
KP63	256	256	256	256	256	256	-	512	256	512	64(< 2)
K2	512	-	512	512	1024	512	-	1024	512	1024	32(<2)
K24	512	1024	512	512	512	-	1024	512	512	1024	16(<2)
P. aeruginosa											
PA01	256	512	512	256	256	512	-	256	512	-	16
PA124	512	1024	512	512	512	512	-	512	512	-	32 (< 2)
P. stuartii											
ATCC29916	128	128	256	1024	128	1024	-	1024	256	-	16
NAE16	128	512	256	256	256	256	1024	512	256	-	64(8)
PS2636	512	512	256	256	256	256	-	256	256	512	32
PS299645	512	1024	1024	512	512	512	-	512	1024	-	32(<2)
E. cloacae											
BM47	128	256	512	1024	256	1024	-	128	512	-	≥256 (<2)
ECCI69	256	512	512	256	256	128	-	256	512	-	≥256(16)
BM67	256	512	512	256	256	512	1024	128	512	1024	128 (32)

The results are the average values from three separate experiments.

(–) MIC > 1024 µg/ml.

Chloramphenicol was used as a reference antibiotic.

MIC was measured in absence and presence of PAβN (in brackets).

BCU IASE CENTRAL UNIVERSITY LIBRARY

Table 11 – Susceptibility of bacteria to plant extracts - MBCs (µg/ml) of methanol extracts *vs.* chloramphenicol

Bacterial strains	*A. hybridus*	*V. hymenolepis*	*L. sativa*	*L. capensis*	*S. edule*	*M. esculenta*	*P. vulgaris*	*C. pepo*	*S. nigrum*	*C. frutescens*	Chloramphenicol
E. coli											
ATCC8739	-	-	-	-	1024	1024	-	512	-	-	64
ATCC10536	1024	-	-	-	-	-	-	1024	-	-	128
W3110	1024	512	256	-	512	-	-	512	-	-	-
MC4100	1024	-	-	-	-	-	-	-	-	-	-
AG100A	-	1024	512	-	-	512	-	-	-	-	-
AG100Atet	-	1024	512	-	-	-	-	-	-	-	-
AG102	-	-	512	1024	-	-	-	-	-	-	-
AG100	256	1024	-	1024	-	512	-	1024	-	-	-
E. aerogenes	-	-	-		-				512		
ATCC13048				1024		1024	-	1024		-	128
EA294	-	-	-	-	-	1024	-	-	-	-	32
CM64	512	-	-	512	512	512	-	-	-	-	-
EA3	1024	512	1024	1024	-	512	-	1024	1024	-	-
EA298	512	1024	1024	-	1024	256	-	256	512	-	-
EA27	-	-	-	-	-	512	-	-	-	-	-
EA289	-	1024	-	512	1024	512	-	-	-	-	-
K. pneumoniae								-			
ATCC11296	-	-	-	1024	-	-	-	256	1024	-	64

BCU IASI - CENTRAL UNIVERSITY LIBRARY

Bacterial strains	*A. hybridus*	*V. hymenolepis*	*L. sativa*	*L. capensis*	*S. edule*	*M. esculenta*	*P. vulgaris*	*C. pepo*	*S. nigrum*	*C. frutescens*	Chloramphenicol
KP55	1024	-	-	-	1024	1024	-	1024	512	-	128
KP63	512	512	-	-	-	-	-	512	1024	-	-
K2	1024	-	-	1024	-	-	-	-	-	-	256
K24	-	-	-	-	1024	-	-	512	-	-	512
P. aeruginosa											
PA01	-	-	-	-	-	-	-	-	-	-	256
PA124	-	1024	-	1024	-	-	-	1024	512	-	-
P. stuartii											
ATCC29916	-	256	-	1024	1024	1024	-	1024	512	-	128
NAE16	-	-	512	-	1024	-	-	1024	-	-	256
PS2636	512		1024	1024	-	1024	-	512	-	-	-
PS299645	-	-	-	-	-	-	-	-	-	-	-
E. cloacae											
BM47	-	1024	512	-	-	-	-	-	1024	-	-
ECCI69	1024	512	-	1024	512	1024	-	1024	512	-	-
BM67	512	1024	-	1024	1024	1024	-	1024	-	-	-

The results are the average values from three separate experiments.

(–) MBC > 1024 µg/ml.

Chloramphenicol was used as a reference antibiotic.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

B.C.U. „M. EMINESCU" IAȘI

In plants, secondary metabolites attract beneficial and repel harmful organisms, serve as phytoprotectants and respond to environmental changes. In animals, such compounds have many beneficial effects including antibacterial and antiviral properties. The classes of secondary metabolites detected in the tested vegetables can somehow provide a preliminary explanation on their activities (Voukeng, Kuete et al., 2012). In general, the phytochemical contents (Table 9) were in accordance with the previous reports for some of the vegetables where data were available (Albone, Gaskin et al., 1984, Brandi, Amagliani et al., 2006, Akubugwo, Obasi et al., 2007). It should however be mentioned that the detection of the bioactive phytochemical classes in a plant is not a guarantee for any biological property, as this will depend on the types of compounds, as well as their concentrations and possible interaction with other constituents.

Solanum nigrum has been shown to possess various activities such as antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective diuretic and antipyretic. Though the exact mechanism of action remains to be elucidated in many cases, few are known about its antibacterial properties. In fact, it has been shown that seeds of *S. nigrum* possess good antimicrobial activity against *E. coli* on solid medium (Kumar, Bagchi et al., 1997). Antibacterial activity of leaves methanol extract of this plant against a panel of MDR Gram-negative bacterial strains is supported by the MIC values varying from 128 to 1024 µg/ml (Table 10). *Solanum nigrum* possesses various compounds that are responsible for diverse

BCU ASHCROFT CENTRAL UNIVERSITY LIBRARY

activities. Among these compounds, solanine (found in all parts of the plant), is its major defence product (Jain, Gupta et al., 2011).

Many reports have also been published about the biological properties of *C. pepo*, but these reports are based on the components of the fruits and the seed's oil (Al-Zuhair, Abd El-Fattah et al., 1997, Nkosi, Opoku et al., 2005).

The results of the phytochemical test on *Phaseolus vulgaris* are in accordance with some other reports (Barkat and Kadri, 2011, Doss and Pugualenthi, 2012). *P. vulgaris* was found to inhibit also the growth of Gram positive bacteria *B. subtilis* (Chaurasia and Saxena, 2012). Amarowicz et al. showed that the acetone extract of *P. vulgaris* contains tannins with good antimicrobial properties against *Listeria monocytogenes* (Amarowicz, Dykes et al., 2008). Therefore, the low antibacterial effects of this plant recorded (generally MIC values at 1024 µg/ml - Table 10) could be due to the multi-drug resistance ability of the studied bacteria.

The antibacterial effects of the extract from *C. frutescens* against *Staphylococcus aureus* as well as *K. pneumoniae* and *P. aeruginosa* have been reported (Koffi-Nevrya, Nangabc et al., 2012). The ethanol extract of this plant was also active against MDR strains of *S. aureus* (Shariati, Pordeli et al., 2010). The present study therefore provides additional information on the antibacterial potential of this plant on MDR Gram-negative bacteria with MICs ranging from 256 to 1024 µg/ml. The antibacterial properties of *S. edule* have already been proved against bacteria of clinical relevance by Ordonez et al. which showed that both

BCU IASI CENTRAL UNIVERSITY LIBRARY

fluid extract and tincture of fruits have "very good" antimicrobial activities against MDR staphylococci and enterococci (Ordoñez, Gomez et al., 2003).

The chloroform extract of *M. esculenta* possess antibacterial activities against *Listeria monocytogenes*, *Vibrio cholerae*, *Shigella flexneri* and *Salmonella typhi* whilst ethanol extract was found active against *P. aeruginosa*, *Corynebacterium diphtheriae* and *V. cholera* (Zakaria, Khairi et al., 2006). The activity of *Amaranthus hybridus* was reported against *E. coli*, *S. typhi*, *K. pneumoniae* and *P. aeruginosa* with MICs ranged between 200 and 755 mg/ml (Maiyo, Ngure et al., 2010). The ethyl acetate extract of *M. esculenta* exhibited activity against *S. aureus* and *B. subtilis* whilst the ethanol extract was found effective against *E. coli* (Dahiya, Sheoran et al., 2010).

The high MIC values observed with chloramphenicol can be explained only taking into account the nonspecific resistance mechanism: active efflux of the toxic compound by pumps belonging to the small multidrug resistance (SMR) proteins family (Pages, Lavigne et al., 2009). The fact that the efflux pump inhibitor - phenyl-arginine-beta-naphthylamide (PAβN) enhances the chloramphenicol antibacterial properties is a clear indication that the tested strains express an active efflux system and that this system is responsible for resistance of the tested bacteria to chloramphenicol. The wide substrate specificity of these pumps, as well as their widespread among bacterial species make us believe that these efflux pumps are also responsible for the extrusion of various active compounds from the plant extract out of bacteria cells,

BCU JAFSU CENTRAL UNIVERSITY LIBRARY

therefore preventing their inhibitory effects. Therefore, the activities of the vegetable as observed herein against MDR strains (with MIC comprised between 128 and 1024 µg/mL) could be considered important, taking into account that edible plants were used. Apart for the extracts of *P. vulgaris* and *C. frutescens* which did not show any MBC below 1024 µg/ml, other values further confirmed the bactericidal effect of the 8 remaining extracts as they were generally less than 4-fold greater than corresponding MIC values (Mbaveng, Ngameni et al., 2008).

2.2.2. Antimicrobial and antibiotic-potentiation activities of some edible plants extracts

Some Cameroonian edible plants used traditionally in the treatment of bacterial infections present antimicrobial activity against MDR bacteria expressing active efflux via the Resistance-Nodulation Cell Division (RND)-type pumps: fruits of *Piper nigrum* L (*Piperaceae*), leaves of *Telfairia occidentalis* Hook. F. (*Cucurbitaceae*) and *Vernonia amygdalina* Del. (*Asteraceae*) and fruits of *Syzygium aromaticum* [L.] Merr & Perry (*Myrtaceae*) (Noumedem, Ştefan et al., 2013).

BCU IASI CENTRAL UNIVERSITY LIBRARY

Phytochemical composition and antibacterial activity of the plant extracts

The results of the qualitative phytochemical analysis showed that each of the tested plant extract contains at least 3 classes of secondary metabolites (Table 12).

The antibacterial activities of the extracts alone and in some cases in combination with PAβN on a panel of 29 Gram-negative bacteria are depicted in Table 13. It appears that the extracts from *P. nigrum* and *V. amygdalina* inhibited the growth of all the twenty nine tested bacterial strains within a concentration range from 32 to 1024 µg/mL. A good spectrum of antibacterial activity was also recorded with the extract of *T. occidentalis*, its inhibitory effects being observed against 27/29 (93.1%) of the tested microorganisms. The lowest MIC value (32 µg/mL) was obtained with the extract of *P. nigrum* on *P. aeruginosa* PA01.

Many secondary metabolites belonging to alkaloids, anthocyanins, anthraquinons, flavonoids, phenols, saponins, sterols, tannins and triterpenes were detected in the tested plant extracts. Several compounds from the investigated classes of phytochemicals were previously reported for their antibacterial activities (Cowan, 1999), and their presence in the tested extracts could explain their antibacterial effects. The differences in bacterial susceptibility to the extracts may be either due to the differences in cell wall composition and/or genetic content of their plasmids (Karaman, Şahin et al., 2003) or to the differences in the composition and

BCU ASI CENTRAL UNIVERSITY LIBRARY

the mechanism of action of the bioactive compounds (Ono, Kashimura et al., 2004).

As shown in Table 12, the three most active plants extracts (*P. nigrum*, *T. occidentalis* and *V. amygdalina*) contain more classes of phytochemicals than the extract from *S. aromaticum*. Each of the three most active plant extracts contains at least four classes of secondary metabolites namely alkaloids, phenols, flavonoids and tannins. However, it should be noted that the activity does not depend on the number of classes of detected bioactive compounds, but mostly on their concentration. The inhibitory activity of *P. nigrum* was previously reported against some bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli* (Pavithra and Bhagya, 2009), and the data reported here confirms the antimicrobial potential of this plant. It has also been demonstrated that the acetone-ethanol extract of the leaves from *V. amygdalina* was weakly active against *K. pneumoniae*, *E. coli*, *S. aureus, B. cereus*, *S. dysentriae* and *S. typhimurium* with MIC values ranged from 7.5 mg/mL to 25 mg/mL (Ibrahim, Ajala et al., 2009). Even if these activities are in accordance with the results obtained, higher antibacterial activity of this plant on all 29 bacteria including MDR phenotypes could be observed (with MIC values ranging between 256 and 1024 µg/mL).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Table 12 – Extraction yields, aspects and phytochemical composition of the plant extracts

Scientific names	Part used	Yield (%)	Secondary metabolites								
			alkaloids	phenols	tannins	terpènes	stéroids	flavonoids	anthraquinones	anthocyanins	saponins
Piper nigrum	Fruits	13.18	+	+	+	-	-	+	+	-	-
Syzygium aromaticum	fruits		+	-	-	-	-	+	-	-	-
Telfairia occidentalis	leaves	11.58	+	+	+	-	-	+	-	+	-
Vernonia amygdalina	leaves		+	+	+	-	+	+	-	-	+

(+): Present;

(-): Absent;

*The yield was calculated as the ratio of the mass of the obtained methanol extract/mass of the plant powder

BCU IASH CENTRAL UNIVERSITY LIBRARY

Table 13 – Minimal Inhibitory Concentration (MIC) in µg/mL of methanol extracts from the four edible plants and chloramphenicol

Bacteria strains	*Piper nigrum*	*Syzygium aromaticum*	*Telfairia occidentalis*	*Vernonia amygdalina*	Chloramphenicol
E. coli					
ATCC8739	128	1024	512	512	4
ATCC10536	256	-	512	512	4
W3110	256	512	256	512	8
MC4100	256	1024	512	512	16
AG100A	128 (64)	-	512 (512)	512 (512)	<2 (<2)
AG100Atet	256 (256)	-	512 (512)	1024 (1024)	64 (<2)
AG102	512 (128)	-	1024 (1024)	256 (256)	64
AG100	256 (32)	-	512 (512)	512 (32)	8 (<2)
E. aerogenes					
ATCC13048	512	1024	512	512	8
EA294	512	-	512	1024	16
CM64	512 (256)	1024	256 (64)	256 (32)	256 (8)
EA3	512 (256)	-	1024 (1024)	512 (512)	256 (128)
EA298	256 (128)	-	512 (512)	256 (256)	64 (<2)
EA27	512 (256)	-	-	1024 (1024)	≥256 (<2)
EA289	512 (256)	1024	512 (256)	256 (256)	≥256 (64)

BCU IASI / CENTRAL UNIVERSITY LIBRARY

BCU WEST CENTRAL UNIVERSITY LIBRARY

Bacteria strains	*Piper nigrum*	*Syzygium aromaticum*	*Telfairia occidentalis*	*Vernonia amygdalina*	Chloramphenicol
K. pneumoniae					
ATCC11296	256	1024	1024	512	8
KP55	256 (64)	-	512 (512)	512 (512)	32 (4)
KP63	1024 (256)	-	256 (256)	1024 (1024)	64 (<2)
K2	512	-	-	1024	32
K24	512	-	512	512	16
P. aeruginosa					
PA01	64	-	512	512	16
PA124	256 (128)	512	512 (512)	1024 (1024)	32 (<2)
P. stuartii					
ATCC29916	128	-	128	256	16
NAE16	512	1024	512	256	64
PS2636	256	-	512	512	32
PS299645	1024 (1024)	-	1024	512	32
E. cloacae					
BM47	128	-	512	1024	≥256
ECCI69	128	-	512	512	≥256
BM67	128 (32)	-	512 (16)	1024 (1024)	128 (32)

(−) MIC > 1024 µg/mL

Role of efflux pumps in the susceptibility of Gram-negative bacteria to the tested extracts and effects of the association of some extracts with antibiotics

Fourteen of the studied MDR bacteria were also tested for their susceptibility to the most active plant extracts (*P. nigrum*, *V. amygdalina* and *T. occidentalis*) in the presence of PAβN at 30 µg/mL. When combined with extracts, PAβN improved the activity (decreases of MIC values) of *P. nigrum* on almost all of the tested MDR strains (13/14 - 92.9%). The efflux pump inhibitor also improved the activity of *T. occidentalis* against *E. aerogenes* CM64, EA 289 and *E. cloacae* BM67 as well as that of *V. amygdalina* against *E. coli* AG100 and *E. aerogenes* EA 289 (Table 13).

In order to evaluate the possible synergistic effects of plants extracts and antibiotics, the extracts from *P. nigrum* and *T. occidentalis* were combined with eleven antibiotics (tetracycline, doxycyclin, ciprofloxacin, norfloxacin, streptomycin, kanamycin, chloramphenicol, erythromycin, ampicillin, cefepime and cloxacillin) separately. As results, synergistic effects were observed with the two extracts and most of the tested antibiotics except ß-lactams (ampicillin, cefepime and cloxacillin) - Tables 14 and 15. At MIC/2 and MIC/5 of the extract from *T. occidentalis*, synergistic effects were observed with 7 of the 11 antibiotics (TET, DOX, CIP, NFX, KAN, CHL, ERY) against the tested MDR bacteria (Table 15).

BCU IASI CENTRAL UNIVERSITY LIBRARY

Table 14 – MIC of different antibiotics after the association of the extract of *Piper nigrum* at MIC/2, MIC/5 against eleven MDR bacteria strains

		Bacterial strains, MIC (μg/ml) of antibiotics in the absence and presence of the extract (PN)											
Antibiotic	Extract conc.	PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	PBSS (%)
TET	0	8	16	256	64	8	16	64	16	8	>256	4	
	MIC/2	4(2)^S	16(1)^I	≤2(≥128)^S	16(4)^S	≤2(>4)^S	4(4)^S	16(4)^S	16(1)^I	8(1)^I	>256	≤2(>2)^S	63,6
	MIC/5	4(2)^S	16(1)^I	≤2(≥128)^S	16(4)^S	≤2(>4)^S	4(4)^S	8(8)^S	16(1)^I	8(1)^I	>256	≤2(>2)^S	63,6
DOX	0	16	8	32	32	32	32	32	32	8	32	64	
	MIC/2	8(2)^S	≤2(>4)^S	≤2(>16)^S	≤2(>16)^S	≤2(>16)^S	4(8)^S	8(4)^S	32(1)^I	≤2(>4)^S	32(1)^I	4(16)^S	81,8
	MIC/5	8(2)^S	≤2(>4)^S	≤2(>16)^S	≤2(>16)^S	4(8)^S	≤2(>16)^S	8(4)^S	16(1)^I	≤2(>4)^S	32(1)^I	≤2(>32)^S	81,8
CIP	0	32	4	128	64	64	128	≤2	8	128	64	128	
	MIC/2	32(1)^I	≤2(>2)^S	16(8)^S	4(16)^S	8(8)^S	64(2)^S	≤2	4(2)^S	64(2)^S	≤2(>32)^S	64(2)^S	90
	MIC/5	32(1)^I	≤2(>2)^S	64(2)^S	16(4)^S	4(16)^S	64(2)^S	≤2	4(2)^S	128(1)^I	≤2(>32)^S	128(1)^I	70
NFX	0	128	128	64	128	≤2	128	32	64	128	128	256	
	MIC/2	64(2)^S	4(32)^S	≤2(>32)^S	16(8)^S	≤2	32(4)^S	8(4)^S	16(4)^S	128(1)^I	16(8)^S	128(2)^S	90
	MIC /5	128(1)^I	≤2(>64)^S	64(1)^I	16(8)^S	≤2	32(4)^S	16(2)^S	16(4)^S	128(1)^I	16(8)^S	128(2)^S	80
STR	0	256	≤2	256	64	8	32	16	64	≤2	4	64	
	MIC/2	256(1)^I	≤2	64(4)^S	≤2(>32)^S	≤2(>4)^S	≤2(>16)^S	32(0.5)^I	4(16)^S	≤2	≤2(>2)^S	≤2(>32)^S	77.8

BCU IASI CENTRAL UNIVERSITY LIBRARY

BCU IADT/CENTRAL UNIVERSITY LIBRARY

Bacterial strains, MIC (µg/ml) of antibiotics in the absence and presence of the extract (PN)													
Antibiotic	Extract conc.	PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	PBSS (%)
	MIC /5	256(1)^{I}	≤2	64(4)^{S}	≤2(>32)^{S}	≤2(>4)^{S}	4(8)^{S}	16(1)^{I}	4(16)^{S}	≤2	≤2(>2)^{S}	≤2(>32)^{S}	77.8
	0	ND	8	128	32	≤2	16	16	32	≤2	8	≤2	
KAN	MIC/2	ND	≤2(>4)^{S}	≤2(>64)^{S}	16(2)^{S}	≤2	≤2(>8)^{S}	4(4)^{S}	4(8)^{S}	≤2	16(0.5)	≤2	75
	MIC /5	ND	≤2(>4)^{S}	≤2(>64)^{S}	4(8)^{S}	≤2	≤2(>8)^{S}	4(4)^{S}	4(8)^{S}	≤2	8(1)^{I}	≤2	75
	0	32	64	>256	64	256	32	>256	>256	64	16	128	
CHL	MIC/2	16(2)^{S}	4(16)^{S}	16(>16)^{S}	64(1)^{I}	32(8)^{S}	4(8)^{S}	32(>8)^{S}	>256	32(2)^{S}	8(2)^{S}	8(16)^{S}	81.8
	MIC /5	32(1)^{I}	16(4)^{S}	16(>16)^{S}	64(1)^{I}	128(2)^{S}	4(8)^{S}	64(>4)^{S}	>256	32(2)^{S}	16	128(1)^{I}	54,5
	0	128	32	256	128	>256	256	64	256	256	256	256	
ERY	MIC/2	128(1)[44]^{I}	8(4)^{S}	64(4)^{S}	256(0.5)^{I}	32(>8)^{S}	64(4)^{S}	32(2)^{S}	256(1)^{I}	64(4)^{S}	16(16)^{S}	64(4)^{S}	72.7
	MIC /5	128(1)^{I}	16(2)^{S}	32(4)^{S}	128(1)^{I}	8(>32)^{S}	256(1)^{I}	8(8)^{S}	256(1)^{I}	128(2)^{S}	16(16)^{S}	64(4)^{S}	63.6
	0	128	128	>256	>256	>256	>256	>256	>256	>256	>256	>256	
AMP	MIC/2	128(1)^{I}	128(1)^{I}	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC /5	128(1)^{I}	128(1)^{I}	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	0	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
FEP	MIC/2	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC /5	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Bacterial strains, MIC (µg/ml) of antibiotics in the absence and presence of the extract (PN)

Antibiotic	Extract conc.	PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	PBSS (%)
	0	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
CLX	MIC/2	>256	16(>16)^{S}	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC /5	>256	32(>8)^{S}	>256	>256	64(>4)^{S}	>256	>256	>256	>256	>256	>256	

Antibiotics: TET: tetracycline, DOX: doxycyclin, CIP: ciprofloxacin, NOR: norfloxacin, STR: streptomycin, KAN: kanamycin, CHL: chloramphenicol, ERY: erythromycin, AMP: ampicillin, FEP: cefepime; CLX: cloxacillin;

PBSS: percentage of bacteria strain on which synergism has been observed;

(): fold increase in MIC values of the antibiotics after association with plants extract;

S: Synergy, I: Indifference.

Table 15 – MIC of different antibiotics after the association of the extract of *Telfairia occidentalis* at MIC/2, MIC/5 against eleven MDR bacteria

		Bacterial strains, MIC (µg/ml) of antibiotics in the absence and presence of the extract											
Antibiotics	Extract conc.	PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	PBSS (%)
	0	8	16	256	64	8	16	64	16	8	>256	4	
TET	MIC/2	$4(2)^S$	$8(2)^S$	$256(1)^I$	$32(2)^S$	$\leq 2(>4)^S$	$\leq 2(>8)^S$	$64(1)^I$	$8(2)^S$	$4(2)^S$	$256(>1)^s$	$\leq 2(>2)^S$	81.81
	MIC/5	$4(2)^S$	$8(2)^S$	$256(1)^I$	$32(2)^S$	$\leq 2(>4)^S$	$\leq 2(>8)^S$	$64(1)^I$	$8(2)^S$	$4(2)^S$	$256(>1)^s$	$4(1)^I$	72.72
	0	16	8	32	32	32	32	32	32	8	32	64	
DOX	MIC/2	$2(8)^S$	$\leq 2(>4)^S$	$16(2)^S$	$4(8)^S$	$4(8)^S$	$\leq 2(>16)^S$	$4(8)^S$	$16(2)^S$	$4(2)^S$	$\leq 2(>16)^S$	$32(2)^S$	100
	MIC/5	$8(2)^S$	$\leq 2(>4)^S$	$32(1)^I$	$8(4)^S$	$\leq 2(>16)^S$	$\leq 2(>16)^S$	$8(4)^S$	$16(2)^S$	$\leq 2(>4)^S$	64(<0.5)	$64(1)^I$	72.72
	0	32	4	128	64	64	128	≤2	8	128	64	128	
CIP	MIC/2	$32(1)^I$	$\leq 2(>2)^S$	$64(2)^S$	$\leq 2(>32)^S$	$16(4)^S$	$\leq 2(>64)^S$	≤2	$\leq 2(>4)^S$	$128(1)^I$	$\leq 2(>32)^S$	$128(1)^I$	70
	MIC/5	$32(1)^I$	$\leq 2(>2)^S$	$64(2)^S$	$\leq 2(>32)^S$	$16(4)^S$	$\leq 2(>64)^S$	≤2	$\leq 2(>4)^S$	$128(1)^I$	$\leq 2(>32)^S$	$128(1)^I$	70
	0	128	128	64	128	≤2	128	32	64	128	128	256	
NFX	MIC/2	$64(2)^s$	$4(32)^S$	$32(2)^S$	$4(32)^S$	≤2	$4(32)^S$	$4(8)^S$	$32(2)^S$	$128(1)^I$	$\leq 2(>64)^S$	$128(2)^S$	90
	MIC /5	$32(4)^s$	$4(32)^S$	$32(2)^S$	$4(32)^S$	≤2	$4(32)^S$	$4(8)^S$	$16(4)^S$	$128(1)^I$	$8(16)^S$	$256(1)^I$	80
	0	256	≤2	256	64	8	32	16	64	≤2	4	64	
STR	MIC/2	$256(1)^I$	≤2	$256(1)^I$	$16(4)^S$	$\leq 2(>4)^S$	$\leq 2(>16)^S$	$16(1)^I$	$\leq 2(>32)^S$	≤2	$\leq 2(>2)^S$	$16(4)^S$	66.67
	MIC /5	$256(1)^I$	≤2	$256(1)^I$	$8(8)^S$	$\leq 2(>4)^S$	$\leq 2(>16)^S$	$16(1)^I$	$\leq 2(>32)^S$	≤2	$\leq 2(>2)^S$	$16(4)^S$	66.67

BCU IASHI CENTRAL UNIVERSITY LIBRARY

BCU WOLFSON CENTRAL UNIVERSITY LIBRARY

Antibiotics	Extract conc.	Bacterial strains, MIC (μg/ml) of antibiotics in the absence and presence of the extract											PBSS (%)
		PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	
KAN	0	ND	8	128	32	≤2	16	16	32	≤2	8	≤2	
	MIC/2	ND	4(2)^{S}	128(1)^{I}	8(4)^{S}	≤2	≤2(>8)^{S}	≤2(>8)^{S}	≤2(>16)^{S}	≤2	8(1)^{I}	≤2	71.42
	MIC /5	ND	4(2)^{S}	128 (1)^{I}	≤2(>16)^{S}	≤2	≤2(>8)^{S}	≤2(>8)^{S}	≤2(>16)^{S}	≤2	8(1)^{I}	≤2	71.42
CHL	0	32	64	>256	64	256	32	>256	64	16	128	128	
	MIC/2	16(2)^{s}	32(2)^{S}	>256	64(1)^{I}	≤2(>128)^{S}	4(8)^{S}	256(>1)^{s}	16(4)^{S}	≤2(>8)^{S}	16(8)^{S}	16(8)^{S}	90
	MIC /5	32(1)^{I}	32(2)^{S}	>256	64(1)^{I}	≤2(>128)^{S}	8(4)^{S}	256(>1)^{s}	32(2)^{S}	16(1)^{I}	64(2)^{S}	64(2)^{S}	70
ERY	0	128	32	256	128	>256	256	64	256	256	256	256	
	MIC/2	64(2)^{s}	16(2)^{S}	>256	32(4)^{S}	≤2	4(64)^{S}	32(2)^{S}	64(4)^{S}	32(8)^{S}	8(32)^{S}	128(2)^{S}	
	MIC /5	64(2)^{s}	32(1)^{I}	>256	32(4)^{S}	64(>4)^{S}	4(64)^{s}	64(1)^{I}	256(1)^{I}	32(8)^{S}	16(16)^{S}	128(2)^{S}	
BAC	0	256	>256	>256	>256	>256	>256	>256	256	>256	>256	>256	
	MIC/2	128(2)^{S}	32(8)^{S}	>256	>256	128(>2)^{S}	128(>2)^{S}	>256	64(4)^{S}	256(>1)^{s}	>256	>256	
	MIC /5	128(2)^{S}	32(8)^{S}	>256	>256	32(>8)^{S}	>256	>256	128(2)^{S}	256(>1)^{s}	>256	>256	
AMP	0	128	128	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC/2	256(0.5)^{I}	128(1)^{I}	>256	>256	>256	256(>1)^{s}	>256	>256	>256	>256	>256	
	MIC /5	256(0.5)^{I}	128(1)^{I}	>256	>256	>256	>256	>256	>256	>256	>256	>256	
FEP	0	ND	>256	>256	>256	>256	>256	>256	>256	256	>256	>256	
	MIC/2	ND	>256	>256	>256	>256	>256	>256	>256	>256(<1)	8(>32)^{S}	>256	

Antibiotics	Extract conc.	Bacterial strains, MIC (µg/ml) of antibiotics in the absence and presence of the extract											PBSS (%)
		PA124	AG100	AG102	AG100Atet	CM64	EA3	EA27	EA289	BM67	KP55	NEA16	
CLX	MIC /5	ND	>256	>256	>256	>256	>256	>256	>256	>256(<1)	>256	>256	
	0	>256	256	>256	>256	>256	>256	>256	>256	>256	>256	>256	
	MIC/2	>256	64(>4)^{S}	>256	>256	256(≥2)^{S}	64(>4)^{S}	>256	>256	>256	>256	>256	
	MIC /5	>256	64(>4)^{S}	>256	>256	256(≥2)^{S}	128(>2)^{S}	>256	>256	>256	>256	>256	

Antibotics - TET: tetracycline, DOX: doxycyclin, CIP: ciprofloxacin, NOR: norfloxacin, STR: streptomycin, KAN: kanamycin, CHL: chloramphenicol, ERY: erythromycin, AMP: ampicillin, FEP: cefepime, CLX: cloxacillin.

Bacterial strains: *Escherichia coli* [AG100, AG100A, AG100Atet], *Pseudomonas aeruginosa* [PA124], *Enterobacter aerogenes* [CM64, EA3, EA27, EA289], *Enterobacter cloacae* [BM67], *Klebsiella pneumoniae* [KP55], *Providencia stuartii* [NAE16].

PBSS: percentage of bacteria strain on which synergism has been observed;

(): fold increase in MIC values of the antibiotics after association with plants extract;

S: Synergy, I: Indifference.

BCU IAMR CENTRAL UNIVERSITY LIBRARY

All the bacterial strains tested with a combination of plant extract and PAβN were proven to possess multidrug resistance efflux pumps (Lomovskaya and Bostian, 2006). Tripartite efflux systems, mainly those clinically described such as AcrAB-TolC in *Enterobacteriaceae* or MexAB-OprM in *P. aeruginosa* play a central role in multidrug resistance of pathogenic Gram negative bacteria (Papadopoulos, Carson et al., 2008). PAßN, a potent inhibitor of the RND efflux systems is especially active on AcrAB-TolC and MexAB-OprM (Pages, Lavigne et al., 2009) and does not present any intrinsic effect on the bacteria at the concentration of 30 µg/mL used in this study (Lorenzi, Muselli et al., 2009). In the presence of PAßN at this concentration, significant increase of the activity of the extract from *P. nigrum* was noted against 13/14 of the tested MDR bacteria. This shows that at least one active compound from this plant, acting inside the bacteria cell could be the substrate of efflux pumps. From this observation, it can be suggested that the association of the extract of *P. nigrum* and efflux pump inhibitors could be helpful in the fight against infections due to MDR bacteria.

A beneficial effect of the combination of two of the tested plant extracts namely those from *P. nigrum* and *T. occidentalis*, with the first line antibiotics could be achieved. Their synergistic effects with antibiotics were noted on more than 70% of the tested MDR bacteria (with seven antibiotics), also suggesting that some of their constituents can act as efflux pump inhibitor (Braga, Leite et al., 2005). This hypothesis is emphasized by the fact that these extracts were more synergistic with antibiotics acting inside the bacteria cells. Besides, it has

BCU IASI / CENTRAL UNIVERSITY LIBRARY

already been proved that the extract from *P. nigrum* can also act by improving the penetration of antibiotics in cells via membrane alteration. However, further phytochemical investigations will permit the isolation of *P. nigrum*, *T. occidentalis* and *V. amygdalina* active constituents. Besides, toxicological studies will be necessary to evaluate their safety.

2.3. ANTIBACTERIAL EFFECT OF NOBLE METAL NANOPARTICLES

In the continuous quest for new antibacterial agent's scenario (Rai, Yadav et al., 2009), nanoscale materials have emerged up as novel antimicrobial agents due to their unique chemical and physical properties (Rai, Yadav et al., 2009). Over the past few decades, inorganic nanoparticles (NPs), whose structures exhibit significantly novel and improved physical, chemical and biological properties due to their nanoscale size, have elicited much interest (Pal, Tak et al., 2007). In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science. Furthermore, nanomaterials can be modified for better efficiency in order to facilitate their applications in different fields such as bioscience and medicine.

BCU IAS / CENTRAL UNIVERSITY LIBRARY

2.3.1. Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media

The antibacterial activity of silver species has been well known since ancient times (Shrivastava, Bera et al., 2007) and it has been demonstrated that in low concentrations silver is non toxic to human cells (Pal, Tak et al., 2007). Silver NPs are substantially more effective than silver ions (Egorova, Revina et al., 2001), enhanced antibacterial properties of silver NPs being demonstrated both *in vitro* and *in vivo* (Rai, Yadav et al., 2009). It is a well known that Ag ions and Ag-based compounds are highly toxic to microorganisms, showing strong antibacterial effects on as many as 12 species of bacteria including *Escherichia coli* and *Staphylococcus aureus* (Zhao and Stevens, 1998, Morones, Elechiguerra et al., 2005, Kim, Kuk et al., 2007, Martínez-Castañón, Niño-Martínez et al., 2008).

The actual bactericide mechanism of silver NPs is not fully understood. Some researchers support the idea that silver is highly reactive, as it binds to tissue proteins and brings structural changes in the bacterial cell wall and nuclear membrane leading to cell distortion and death (Marini, De Niederhausern et al., 2007). Silver also binds to bacterial DNA and RNA by denaturing and inhibits bacterial replication (Castellano, Shafii et al., 2007). Morones et al. (2005) defined the antibacterial activity of silver NPs against some Gram negative bacteria

BCU "ASE" CENTRAL UNIVERSITY LIBRARY

and suggested that silver NPs attach to the surface of the cell membrane, disturb its function, penetrate bacteria, and release silver ions (Morones, Elechiguerra et al., 2005).

The size-dependent interaction of silver NPs with bacteria has been reported by previous studies (Morones, Elechiguerra et al., 2005, Martínez-Castañón, Niño-Martínez et al., 2008). However, little is known about the correlation between the shape of silver NPs and their biological activity (Pal, Tak et al., 2007).

Some authors reported that silver NPs used as drug disinfectant have some risks as the exposure to silver can cause agyrosis and argyria; also in high concentrations are toxic to mammalian cells (Gong, Li et al., 2007). Therefore it is essential to investigate the antimicrobial effects of low concentrations of Ag NPs against representative microorganisms of public health concern. Different Ag NPs sizes and concentrations can be applied effectively in the control of microorganisms and the prevention of deleterious infections. Also Ag NPs can be prepared in a simple and cost-effective manner and are suitable for formulation of new types of bactericidal materials (Stefan, Hritcu et al., 2011).

Silver NPs characterization

The synthesis of Ag-PAmHU coated NPs was performed in a simple two-electrode cell using an Amel 549 potentiostat/galvanostat. An aqueous solution obtained from AgClO4 (10-4 M) and poly(amide-hydroxyurethane)-PAmHU polymer (0.1% w/w) was used as supporting

RGU IAST CENTRAL UNIVERSITY LIBRARY

electrolyte; pH was adjusted to 10 with a carbonate and bicarbonate mixture solution.

The characteristic TEM micrographs of the two types of Ag (Ag I and Ag II) NPs-PAmHU systems show that the Ag NPs tend to be agglomerated inside the PAmHU matrix. The particles were found to be spherical or polyhedral in shape, having an average size of 28.87 nm (± 9.16 nm) for Ag I and 23 nm (±11.13 nm) for Ag II. On the other hand the LG analysis revealed that the average size of Ag I is 43.79 nm and respectively 40.68 nm for Ag II due to NPs packing in a thick layer of PAmHU. All Ag-capped PAmHU colloidal solutions have a moderate stability (APZ within –40 to –30 mV).

Effect of silver nanoparticles on bacterial growth

Since Klabunde and co-workers proved that reactive metal oxide NPs present excellent bactericidal effects (Stoimenov, Klinger et al., 2002), it is of great interest to investigate the use of other inorganic NPs as antibacterial materials. These inorganic NPs have a distinct advantage over conventional chemical antimicrobial agents (Kim, Kuk et al., 2007). The most important problem caused by the chemical antimicrobial agents is multidrug resistance. An alternative way to overcome the drug resistance of various microorganisms is needed, especially in medical devices, etc. Ag ions and Ag salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms (Marambio-Jones and Hoek, 2010).

BCU JASTI CENTRAL UNIVERSITY LIBRARY

In order to asses the effectiveness of silver NPs as antimicrobial agents, an initial disk diffusion assay was performed against two representative microorganisms: Gram negative bacterium *Escherichia coli* (ATCC 25922) and Gram positive bacterium *Staphylococcus aureus* (ATCC 25922). The experimental data (Fig. 24) indicates that, regardless of the size, the Ag NPs produced an inhibition zone on the Muller-Hinton agar plates for both tested bacteria.

S. aureus – Ag I

E. coli – Ag II

Fig. 24 – Disk diffusion assay using Muller-Hinton agar supplemented with 29 nm (Ag I) respectively 23 nm (Ag II) at different concentrations: 1 - 0.25 µg Ag /disk, 2 - 0.75 µg Ag /disk, 3 -1.50 µg Ag /disk

A clear connection between the inhibition zone diameter and silver concentration can be inferred. Similar results were previously reported by

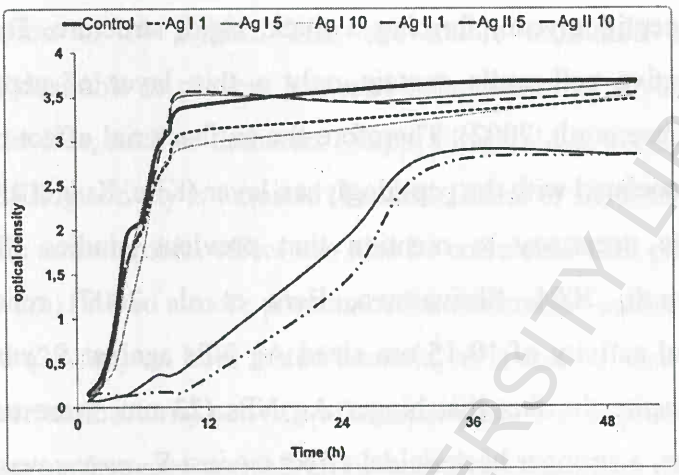
BCU IASI CENTRAL UNIVERSITY LIBRARY

Shrivastava et al. using a plate culturing method and incorporating silver NPs in the growth medium (Shrivastava, Bera et al., 2007).

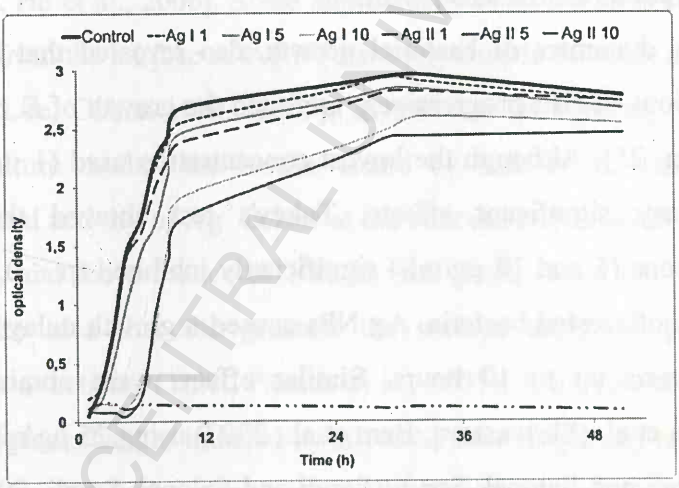
Although growth on agar plates is a more ready means of distinguishing antimicrobial properties of Ag NPs of different sizes, Sondi et al. previously reported different effects of Ag NPs depending on growth conditions used (Sondi and Salopek-Sondi, 2004). Thereby liquid-growth experiments must be employed. Because most of the studies describing antibacterial activity of silver NPs reported in the literature were performed at high concentrations (10, 50, 100 μg/mL (Sondi and Salopek-Sondi, 2004, Cho, Park et al., 2005, Morones, Elechiguerra et al., 2005), further experiments were carried out using LB medium supplemented with low concentrations of Ag NPs: 1, 5 and 10 μg/mL.

PAmHU coated Ag NPs showed antimicrobial activity against both Gram positive and Gram negative tested bacteria (Fig. 25). Nevertheless, distinct reactions between the two microorganisms could be observed, results being in line with previous reports by several authors (Sondi and Salopek-Sondi, 2004, Kim, Kuk et al., 2007, Shrivastava, Bera et al., 2007). The inhibitory effect of Ag NPs was mild in the case of *S. aureus* as compared with *E. coli* and was determined only by Ag II. Ag I had no significant influence on *S. aureus* growth regardless of tested concentrations. Both types of used NPs significantly inhibited the development of *E. coli* cells ($p<0.0001$). The results suggest that the antimicrobial effects of Ag NPs could be associated with characteristics of certain bacterial species.

BCU IASI / CENTRAL UNIVERSITY LIBRARY



a



b

Fig. 25 – Bacterial dynamic growth curve in LB media at different sizes (Ag I - 29 nm, Ag II - 23 nm) and concentrations (1, 5 and 10 µg Ag): a – *S. aureus*; b – *E. coli*

The lower efficacy of the Ag NPs against *S. aureus* may derive from the differences in cell wall structure and permeability compared with *E. coli*. In most Gram positive bacteria, the cell wall consists of many

BCU IASI / CENTRAL UNIVERSITY LIBRARY

layers of peptidoglycan, forming a thick, rigid, structure. By contrast, Gram negative cell walls contain only a thin layer of peptidoglycan (Tortora, Case et al., 2002). Therefore the antibacterial effect of Ag NPs must be associated with the peptidoglycan layer (Kim, Kuk et al., 2007).

It is necessary to mention that previous studies (Sondi and Salopek-Sondi, 2004, Shrivastava, Bera et al., 2007) reported low antibacterial activity of 10-15 nm sized Ag NPs against *S. aureus* at 25 μg/mL. Despite the fact that bigger Ag NPs (23 nm) were used in the experiments, a stronger bactericidal effect against *S. aureus* was observed at concentrations as low as 5 μg/mL.

The dynamics of bacterial growth also revealed that increasing concentrations of NPs progressively inhibited the growth of *E. coli* and *S. aureus* (Fig. 25). Although the lowest concentration used (1 μg/mL) did not had any significant effects, Tukey's test showed that higher concentrations (5 and 10 μg/mL) significantly inhibited ($p < 0.0001$) the growth of both tested bacteria. Ag NPs caused a growth delay of *E. coli* and *S. aureus* up to 10 hours. Similar effects were obtain also by Shrivastava et al. (Shrivastava, Bera et al., 2007) using 25 μg/mL Ag NPs and by Sondi and Salopek-Sondi (Sondi and Salopek-Sondi, 2004) using 50 and 100 μg/mL Ag NPs final concentration in the growth medium. The lag phase was found to be more prolonged although lower concentrations of Ag NPs (5, 10 μg/mL) were used. This could be explained by the greater stability of the PAmHU coated NPs used. Moreover, it is necessary to emphasize that Ag II have bactericidal effects resulting not only in inhibition of bacterial growth but also in killing *E. coli* cells at a

BCU JASSY CENTRAL UNIVERSITY LIBRARY

concentration of 10 μg/mL. This irreversible inhibition of bacterial growth is desirable to prevent bacterial colonization of silver-containing medical devices, such as catheters (Rupp, Fitzgerald et al., 2004), where bacteria-killing activity is required (Panácek, Kolár et al., 2009).

The antimicrobial effect can also be correlated with particles size: Ag II (23 nm) proved a more pronounced antimicrobial activity compared with Ag I (29 nm) on both tested bacteria, as it can be seen in Fig. 25. This higher antibacterial activity of smaller sized NP is probably due to their bigger surface area and therefore their greatest contact with bacterial cell (Lok, Ho et al., 2006). Some authors argue that for solid systems Ag^+ released from the surface of Ag NPs are responsible for their antibacterial activity (Lee, Cohen et al., 2005). In the case of aqueous systems (as liquid culture media) the results found by Lok et al. show that the antibacterial activity of Ag^+ is low at the concentrations levels reached by releasing, and the presence of NPs is vital (Lok, Ho et al., 2006). This proves the idea that the greatest the surface area, the greatest the antibacterial activity (Thiel, Pakstis et al., 2007). Although structural and morphological changes induced by silver NPs are frequently reported (Rai, Yadav et al., 2009), the mechanism of the bactericidal effect of Ag NPs is partially known. It is accepted that Ag NPs attach to the cell surface and may disturb the permeability and respiration functions (Kvitek, Panaček et al., 2008).

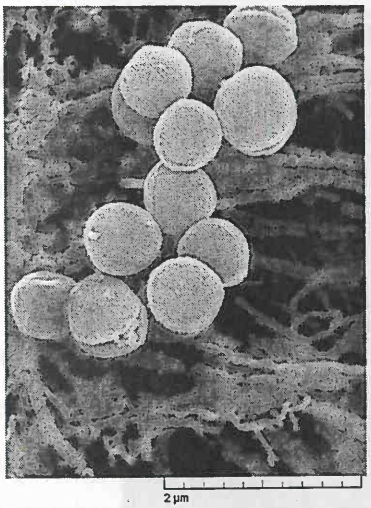
In order to demonstrate that the Ag NPs have a direct contact with bacteria, as well as a potential for inducing changes in bacterial cell morphology, SEM microscopy was employed. A significant number of

BCU IASI / CENTRAL UNIVERSITY LIBRARY

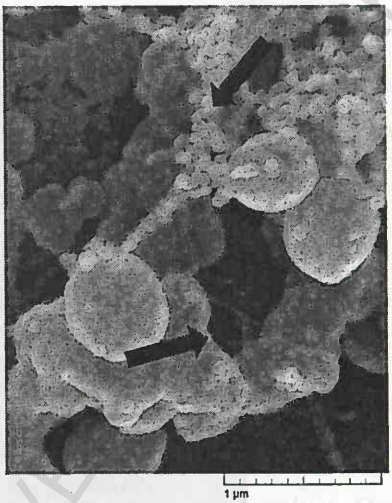
NPs that have been released from the PAmHU matrix and clusters of NPs both types anchored on the surface of bacteria membrane was observed. The Ag NPs treated bacterial cells showed major damage and their morphology was significantly changed (Fig. 26). Clusters of NPs were found to anchor to the bacterial cell wall (Fig. 27), possibly at sites that are rich in negatively charged functional groups.

The overall surface of the bacteria is negatively charged at biological pH values, due to the dissociation of functional groups (carboxylic and other) in the membrane (Stoimenov, Klinger et al., 2002). In view of the strongly electronegative character of the surfaces of cells, their capacity for binding anionic NPs-polymer aggregates is unexpected. Nevertheless, the NPs-PAmHU aggregates are heterogeneous mixtures of various NPs differing widely in size, degree of PAmHU-NPs coated, and fractions of charged surface functional groups. As result charged functional groups from cell membrane exhibits any degree of selectivity for different fractions of polymer surface functional groups. The highly electronegative potential of the three different fractions of PAmHU coated NPs cause a transfer of charge (electrons) and therefore a high reactivity at the interface PAmHU/bacteria membrane. As a result a Donnan equilibrium is restored in the contact area; partial fixed charge may become positive (due to the electron transfer) and therefore favoring the formation of channels with high mobility for the silver ions transport.

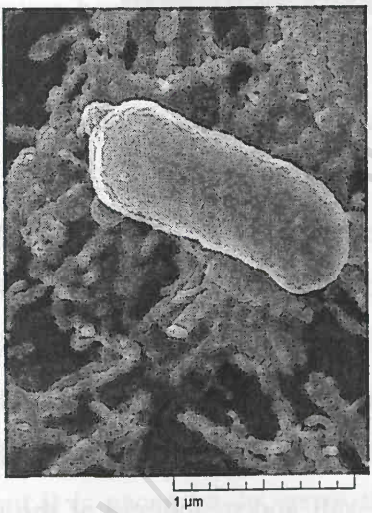
BCU IASI / CENTRAL UNIVERSITY LIBRARY



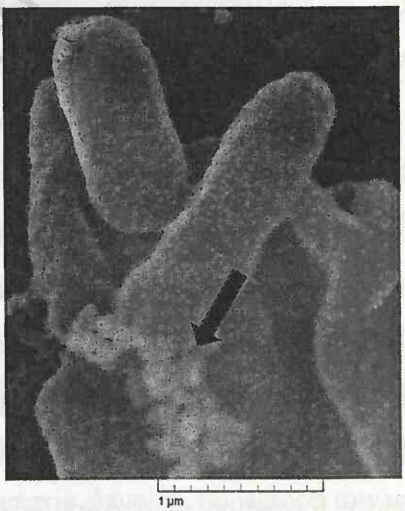
control



Ag II 10 µg/ml



control



Ag II 10 µg/ml

Fig. 26 – SEM micrographs showing the effects of silver NPs on *S. aureus* (up) and *E. coli* (down) cells morphology (arrows indicate the NPs attached on cell wall surface)

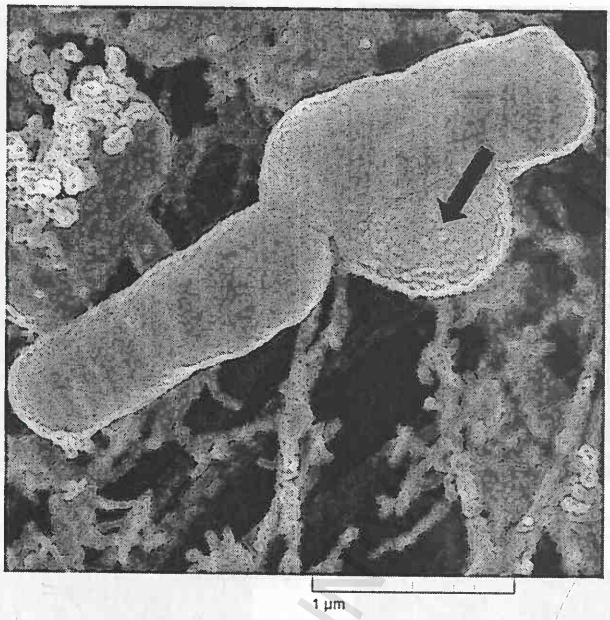


Fig. 27 – SEM micrograph of *E. coli* cells and NPs aggregates

The Gram negative bacteria have a layer of lipopolysaccharide at the exterior, exposing more negative charges on their cell wall then Gram positive bacteria (Tortora, Case et al., 2002). This can explain the results and the observed fact that the growth of *E. coli* was more profoundly affected by the silver NPs than *S. aureus*. These NPs clusters probably induce formation of perforations in the wall and membrane of the cell, which could result in cell lysis (Shrivastava, Bera et al., 2007). It is also possible that Ag NPs not only interact with the cell surface, but can also penetrate inside the bacteria (Morones, Elechiguerra et al., 2005). The damage to cell may be caused by interaction of Ag NPs with phosphorous- and sulfur-containing compounds such as DNA

BCU IASI CENTRAL UNIVERSITY LIBRARY

(Marambio-Jones and Hoek, 2010). Silver tends to have a high affinity for such compounds. Ag^+ strongly interact with the available –SH groups of the biomolecules to inactivate the bacteria. Such interactions in the cell membrane would prevent DNA replications, which would lead to bacterial death (Matsumura, Yoshikata et al., 2003).

Silver NPs used in the study were synthesized in a simple and cost-effective manner and were found to have a stronger antibacterial effect at lower concentrations than those described in the earlier reports (Sondi and Salopek-Sondi, 2004, Shrivastava, Bera et al., 2007). The effect was dose dependent and was more pronounced against Gram negative bacteria than Gram positive ones. Moreover, the effect was size dependent, the smallest Ag NPs used having bactericidal effects resulting in killing *E. coli* cells.

The major mechanism through which silver NPs manifested antibacterial properties was by forming clusters and anchoring to the bacterial cell surface. However, further investigations are necessary for a better understanding of interaction between silver NPs and bacteria components in order to explain the action mechanisms of this nanomaterial. Also, before proposing the use of Ag NPs as antimicrobial agent it is necessary to verify if the bacteria develop resistance towards NPs and to examine cytotoxicity of NPs towards human cells before proposing their therapeutic use (Braydich-Stolle, Hussain et al., 2005).

BCU INSTITUTIONAL UNIVERSITY LIBRARY

2.3.2. Antimicrobial properties of chitosan capped gold nanoparticles

Although gold is the subject of one of the most ancient themes of investigation in science, its renaissance now leads to an exponentially increasing number of publications, especially in the context of emerging nanoscience and nanotechnology with NPs and self-assembled monolayers (Daniel and Astruc, 2003). Due to the strong and size-tunable surface plasmon resonance, fluorescence, and easy-surface functionalization, gold nanoparticles (AuNPs) have attracted increasing interest in diagnosis and therapy of disease, being widely used in biosensors, cancer cell imaging, photothermal therapy and drug delivery (Zhang, Guo et al., 2009). Metals have been used for centuries as bactericidal and bacteriostatic agents (Elsome, Hamilton-Miller et al., 1996). Inorganic NPs such as silver and gold with antimicrobial activity are emerging as a new class of biomedical materials to fulfill the increasing problem regarding antibiotics resistance, being used extensively in many bactericidal fields (Fu, Vary et al., 2005). Their predominant antimicrobial activity can be attributed to the strong cytotoxicity to various bacterial cells; they can interact with the functional groups on the bacterial cell surface and inactivate bacteria (Zhang, Peng et al., 2008). The antibacterial activity of AuNPs depends on their contact surface; a larger surface area of the nanoparticles allows a broader spectrum of interactions with other organic and inorganic molecules

BCU IASI / CENTRAL UNIVERSITY LIBRARY

(Hernández-Sierra, Ruiz et al., 2008). However the question of the mechanisms governing antibacterial action of AuNPs or polymers remains unanswered.

Many dispersed metallic nanoparticles often tend to congregate and separate from solutions, which results in the decrease of their antimicrobial efficiency (Zhang, Peng et al., 2008). Due to the strong reactivity of free electrons present on their surface, uncoated AuNPs are very sensitive to environmental factors such as pH, temperature, electrolytes and solvent, having the tendency to easily aggregate when used in these media. Therefore, how to design AuNPs with the long-term dispersion stability and excellent antimicrobial efficiency has become a primary challenge. In recent years, many kinds of polymers have been investigated as protective agents to stabilize AuNPs and avoid their aggregation (Daniel and Astruc, 2003, Morones, Elechiguerra et al., 2005, Lee, Park et al., 2007). Being readily available, natural polymers (gelatin, dextran, chitosan) have been the first and probably the most frequently used additives for this purpose (Mandal, Fleming et al., 2002). Chitosan is a well-known biopolymer, which displays excellent biocompatibility, nontoxicity, and biodegradability, and is successfully used in nanomedicine in delivering drugs, proteins, and genes (Kean and Thanou, 2010). Previously studies reported AuNPs stabilization with chitosan (Esumi, Takei et al., 2003). Wang et al. demonstrated that chitosan can act not only as a stabilizer but also as a reducer of the gold salt, determining by itself the formation of zero-valent nanoparticles (Wang, Hu et al., 2006). Thus, AuNPs with different size distributions have been

BOUMERDES CENTRAL UNIVERSITY LIBRARY

obtained using chitosan with different molecular weights as an agent of stabilization/reduction (Boca, Potara et al., 2011).

Effect of gold nanoparticles on bacterial growth

In order to asses the effectiveness of AuNPs as antimicrobial agents, an initial well diffusion assay was performed against *E. coli* (Gram negative bacterium) and *S. aureus* (Gram positive bacterium). Regardless of the NPs size and concentration used no inhibition zones could be observed in the case of both tested bacteria.

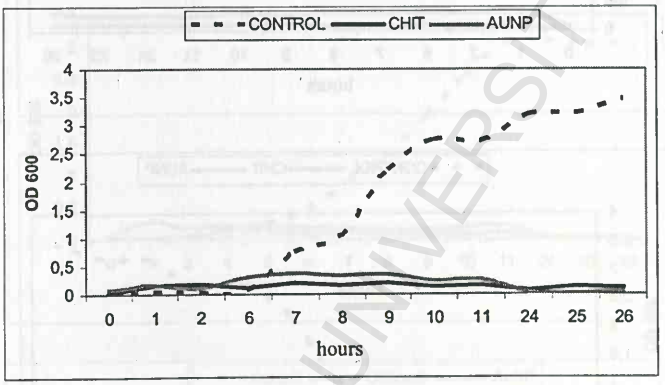
Because previous studies reported different effects of NPs depending on growth conditions used (Sondi and Salopek-Sondi, 2004), further investigations using liquid-growth experiments were employed.

The growth of tested bacteria was inhibited by chitosan and chitosan capped AuNPs (Fig. 28-31), similar antimicrobial effects being observed against both Gram negative and Gram positive bacteria. The recorded differences were always statistical significant as compared with a control (MHB without chitosan and AuNPs).

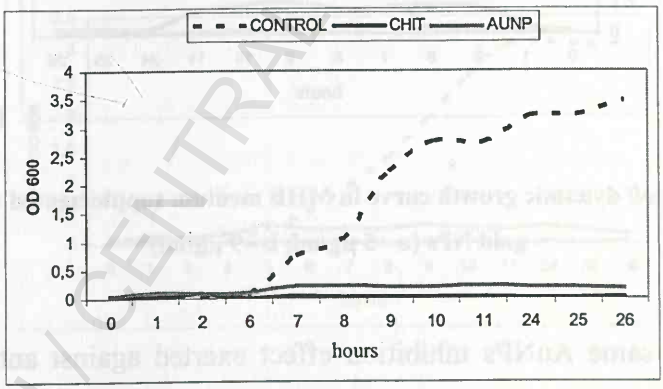
The dynamics of bacterial growth also revealed a different antimicrobial effect registered for chitosan and chitosan capped AuNPs. In the case of *E. coli* a stronger effect was observed when chitosan alone was used to supplement the culture medium compared with a mild effect registered for AuNPs coated with the same polymer. Post-hoc Tukey analysis showed that the recorded differences were statistical significant regardless of AuNPs size for both 9 nm AuNps ($p<0.0001$) and 14 nm

BCU IASI / CENTRAL UNIVERSITY LIBRARY

AuNps ($p<0.0001$) at the highest concentration used (9 µg/ml). It may be presumes that the presence of AuNPs inhibited in this case the antimicrobial effect of chitosan, the inhibition being more pronounced when bigger AuNPs were used (Fig. 28 and Fig. 29).



a



b

Fig. 28 – *E. coli* dynamic growth curve in MHB medium supplemented with 9 nm gold NPs (a - 5 µg/ml, b - 9 µg/ml)

BCU IASI / CENTRAL UNIVERSITY LIBRARY

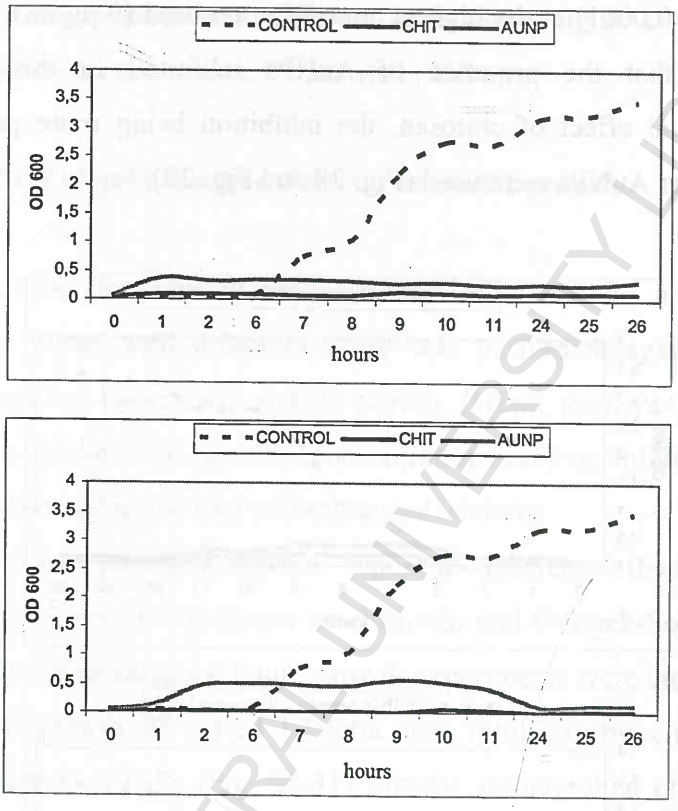
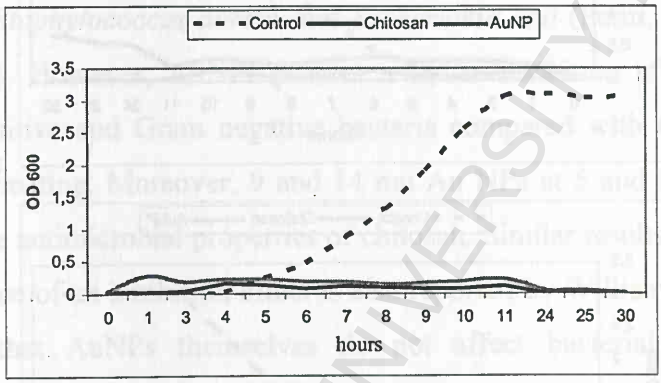


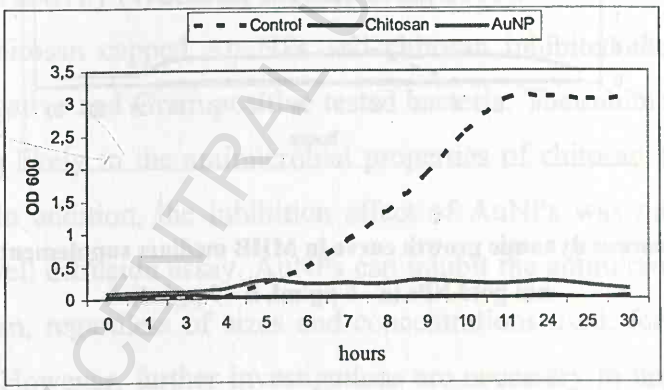
Fig. 29 – *E. coli* dynamic growth curve in MHB medium supplemented with 14 nm gold NPs (a - 5 µg/ml, b - 9 µg/ml)

The same AuNPs inhibition effect exerted against antimicrobial properties of chitosan was revealed when *S. aureus* was used as a test bacterium (Fig. 30 and Fig. 31). Regardless of the NPs sizes and concentrations, the recorded differences between chitosan and chitosan capped AuNPs were always statistical significant ($p<0.0001$) as revealed by Tukey post-hoc analysis.

BCU IASI / CENTRAL UNIVERSITY LIBRARY



a



b

Fig. 30 – *S. aureus* dynamic growth curve in MHB medium supplemented with 9 nm gold NPs (a - 5 µg/ml, b - 9 µg/ml)

BCUIASI / CENTRAL UNIVERSITY LIBRARY

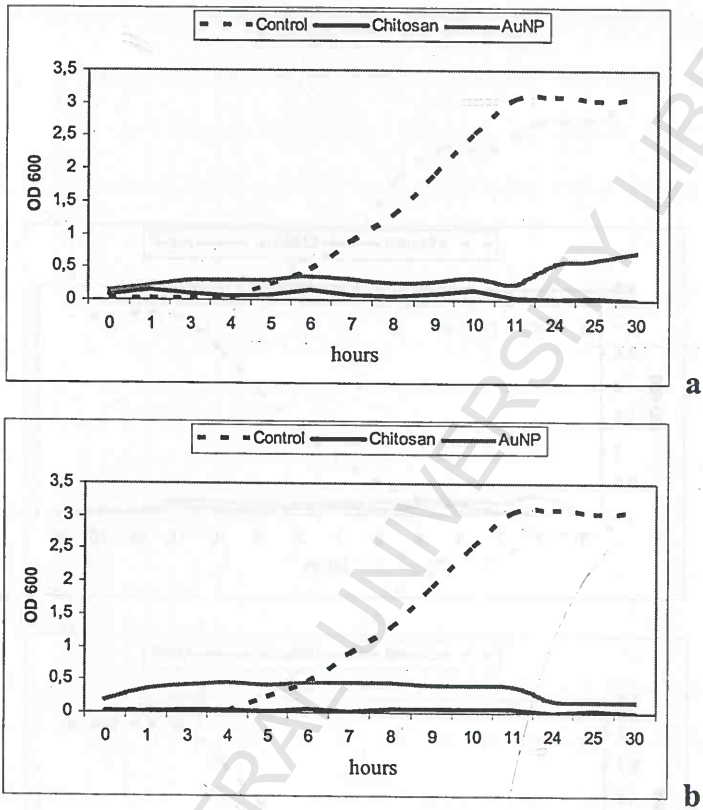


Fig. 31 – *S. aureus* dynamic growth curve in MHB medium supplemented with 14 nm gold NPs (a - 5 µg/ml, b - 9 µg/ml)

Antimicrobial activity of chitosan was previously reported, the growth of *Escherichia coli* being inhibited in the presence of more than 0.025% chitosan (Roller and Covill, 1999, Helander, Nurmiaho-Lassila et al., 2001, Kong, Chen et al., 2010). The cationic amino groups of chitosan probably bind to anionic groups of this microorganism, resulting in growth inhibition (Majeti N.V, 2000). Also, toxicity and antibacterial

BCU IASI CENTRAL UNIVERSITY LIBRARY

effect of AuNPs is well documented (Goodman, McCusker et al., 2004, Hernández-Sierra, Ruiz et al., 2008, Zhang, Peng et al., 2008)

In adition, the presence of nanogold enhances the antibacterial activity of some polymers which are exceptional at killing methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* (Perni, Piccirillo et al., 2009). However, AuNPs present a lower inhibition effect against Gram positive and Gram negative bacteria compared with the chitosan used for coating. Moreover, 9 and 14 nm Au NPs at 5 and 9 μg/ml can inhibit the antimicrobial properties of chitosan. Similar results concerning the absence of an inhibition effect is also reported by Williams et al. who showed that AuNPs themselves do not affect bacterial growth or functional activity (Williams, Ehrman et al., 2006).

Chitosan capped Au NPs and chitosan inhibited the growth of Gram negative and Gram positive tested bacteria. The inhibition effect is due more likely to the antimicrobial properties of chitosan rather to the AuNPs. In addition, the inhibition effect of AuNPs was not evidenced using a well diffusion assay. AuNPs can inhibit the antimicrobial activity of chitosan, regardless of sizes and concentrations used, for both tested bacteria. However, further investigations are necessary to understand the mechanisms involved in this inhibition effect.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Chapter 3.

NEW BIOTECHNOLOGICAL APPLICATIONS OF THE METABOLIC DERIVATIVES FROM *ARTHROBACTER NICOTINOVORANS*

Marius MIHĂȘAN, Lucian HRIȚCU, Marius ȘTEFAN

BCU IAȘI / CENTRAL UNIVERSITY LIBRARY

Introduction

Formerly known as *Arthrobacter oxydans*, the actinobacterium *Arthrobacter nicotinovorans* received its current name in accordance to its most studied property: the ability to metabolize nicotine as carbon and nitrogen source. Biodegradation of this alkaloid is based on the catabolic machinery encoded by pAO1 - a 165 kb megaplasmid (Brandsch and Decker, 1984). The complete sequence of pAO1 (GenBank: AJ507836.1) plasmid was determined and two putative pathways could be described (Igloi and Brandsch, 2003): on one hand the nicotine-degrading pathway, fully characterized by Brandsch (Brandsch, 2006) and on the other hand an yet unknown putative sugar-catabolic pathway.

Even though the metabolic pathway in *A. nicotinovorans* was described in detail, until now there are no biotechnological applications of the identified metabolic products. Therefore, an important goal is to discover new biotechnological applications of some metabolic derivatives from *Arthrobacter nicotinovorans* cells: enzymes needed for efficient use of lignocellulose (Mihăşan, Ştefan et al., 2013) and nicotine metabolites as novel neuroprotective drugs (Mihăşan, Ştefan et al., 2013, Hriţcu, Ştefan et al., 2013).

Lignocellulose provides a promising alternative for replacing oil-based chemicals and fuels due to its high abundance and renewable character. Since the D-xylose fraction of lignocellulose is rather significant, making up to 20% of its biomass, it is essential that this fraction be efficiently utilized to obtain cost-effective biochemical

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

production (Meijnen, de Winde et al., 2009). Although, theoretically, D-xylose can be readily exploited as a valuable resource for production of ethanol and various chemicals, in practice, such applications are scarce mainly due to the lack of suitable enzymes. Enzymes with a broader optimal pH and temperature and better solvent stability are needed for efficient use of D-xylose in biotechnological process development. Such enzymes could be produced by species of soil bacteria occupying an ecological niche rich in lignocellulosic materials such as *A. nicotinovorans* - a very suitable candidate for finding new degradation pathways and new xylose-degrading enzymes. Indeed, pAO1 megaplasmid of *A. nicotinovorans* encodes an oxidative xylose-catabolic pathway. By channeling the D-xylose not only through the pentose phosphate pathway, but also through the Tricarboxylic Acids Cycle (TCA cycle), this supplementary pathway would allow the pAO1 harboring bacteria a more flexible metabolism and thus gain an advantage in environments limited in carbon sources. The sugar-catabolic pathway is comprised of several genes, among which a putative cellulase, an ABC transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases. This last cluster probably encodes the last steps of the pathway, connecting it to the general metabolism of the cell. All of these genes are clearly organized as a single operon, with *orf32*, a putative DNA binding-protein having an opposite orientation and thereby being the perfect candidate for the repressor involved in the control of the whole operon. Three genes of the putative enzymes implicated in this pathway were cloned, purified and characterized (Mihăşan, Ştefan et al., 2012).

BCU IASI CENTRAL UNIVERSITY LIBRARY

Degradation of nicotine (the main alkaloid produced by tobacco plant) in *A. nicotinovorans* is also based on the catabolic machinery encoded also by pAO1 megaplasmid. The degradation starts with the hydroxylation of the pyridine ring to 6-hydroxy-L-nicotine (6HLN). Furthermore, nicotine metabolism is controlled by nicotine metabolite 6-hydroxy-nicotine (6HN), which interacts with 6-hydroxy-nicotine oxidase repressor (HdnoR), disrupting it's binding to the specific operator site and inducing the production of downstream metabolic enzymes.

Numerous studies focused primarily on the neuropharmacological effects of nicotine indicated an important impact of this alkaloid and his metabolites on brain functions. Thus it has become increasingly important to obtain more information on the biological effects of nicotine metabolites derived from mammalian or bacterial cells, in order to possibly manage neurological abnormalities in some neurodegenerative afflictions. Therefore, some studies were designed to determine whether 6HLN, a nicotine metabolite derived from nicotine catabolism in *A. nicotinovorans*, affects spatial memory formation and antioxidant status (Hrițcu, Ştefan et al., 2013).

3.1. EVIDENCE OF A PLASMID-ENCODED OXIDATIVE XYLOSE CATABOLIC PATHWAY IN *ARTHROBACTER NICOTINOVORANS* pAO1

An oxidative xylose catabolic pathway was evidenced on pAO1 megaplasmid of *A. nicotinovorans* and three genes of three putative

BCUJAN CENTRAL UNIVERSITY LIBRARY

enzymes implicated in this pathway were cloned, purified and partially characterized. Also a virtually identical gene cluster on the *A. phenanthrenivorans* chromosome was found, suggesting that a process of horizontal gene transfer among these two species of *Arthrobacter* had happened somewhere in their evolution (Mihasan, Stefan et al. 2013).

General organization of the putative carbohydrate pathway on pAO1

The 165 kb megaplasmid pAO1 of *Arthrobacter nicotinovorans* consists of 165 known or predicted ORFs, 47.7% being related to nutrient utilization. More than half of these ORFs are related to nicotine metabolism and the synthesis of cofactors required by enzymes in this pathway. The rest of the ORF are related to carbohydrate utilization and apparently, form three distinctive groups (Fig. 32).

The first group is located close to the arbitrary-chosen origin of the pAO1 meplasmid and consists of 5 ORFs encoding a LipG related putative hydrolase, a second putative hydrolase, a lipase (esterase), an inositol transport protein and a putative LacI family transcriptional regulator. The smallest group contains only 3 reading frames: a beta-glucosidase, a putative major facilitator superfamily (MFS) transporter and a LacI family transcriptional regulator and is placed close to the *nic*-gene cluster.

BCU JASI CENTRAL UNIVERSITY LIBRARY

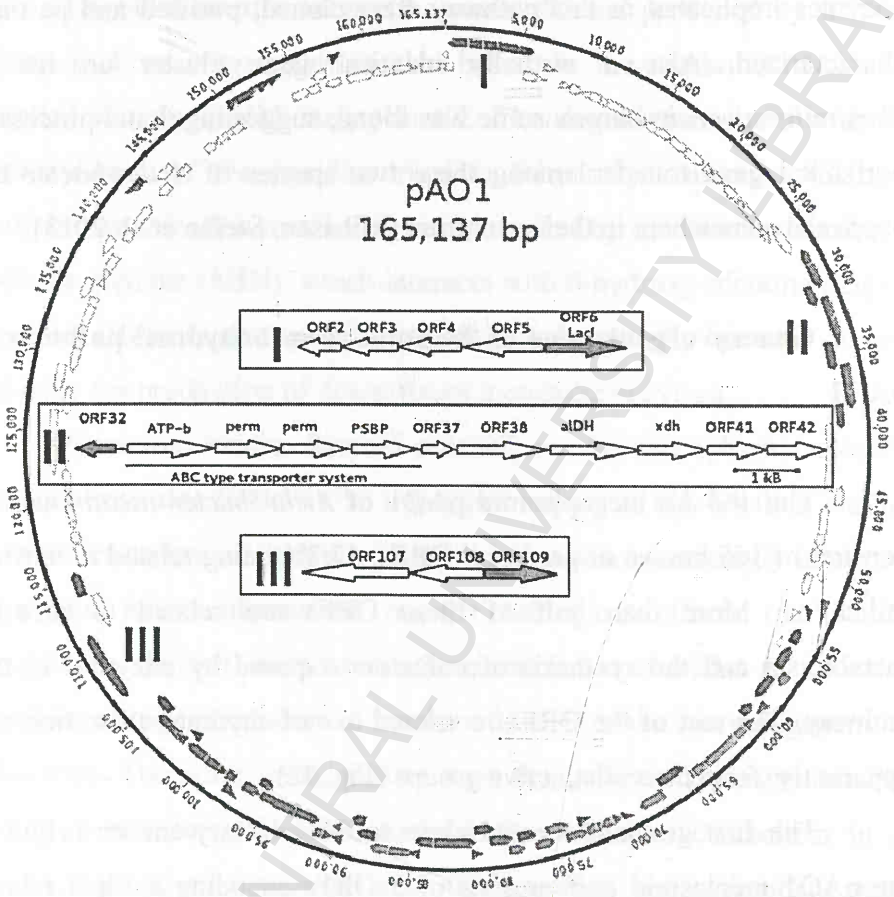

pAO1
165,137 bp
ORF2 ORF3 ORF4 ORF5 ORF6 LacI
ORF32 ATP-b perm perm PSBP ORF37 ORF38 alDH xdh ORF41 ORF42
ABC type transporter system
1 kB
ORF107 ORF108 ORF109

Fig. 32 – Schematic diagram of the pAO1 megaplasmid of *A. nicotinovorans*. Arrows denote ORFs; Shadows of grey indicate ORFs related to carbon metabolism (nicotine and carbohydrate). ORFs related to carbohydrate utilization are grouped into three clusters marked with Roman numerals, their genes arrangement being depicted in the center

By far the largest carbohydrate-related cluster is formed by 11 ORFs starting at nucleotide position 27948. It contains an ORF

BCU IAȘI / CENTRAL UNIVERSITY LIBRARY

homologus to the GntR family transcription factors with an opposite orientation, 4 ORFs of an ATP-binding cassette (ABC) type transporter system: a putative ATP-binding protein, two pore-forming membrane proteins and a periplasmic substrate-binding protein. Downstream from these ORFs are present the ORFs of several oxidoreductases and dehydrogenases: ORF38 (GI:25169060) – coding for an putative 2-keto-gluconate dehydrogenase, ORF39 (GI:25169061) – coding for an aldehyde dehydrogenase, ORF40 (GI:25169062) – encoding a sugar-dehydrogenase, ORF41 (GI:25169063) – a putative conserved protein similar to DUF993. The cluster ends with ORF42 (GI:25169064) – encoding for a protein related to glycerate-kinase (Igloi and Brandsch, 2003). Based on the arrangement and proposed functions of the ORFs, one would suggest a catabolic pathway in which the substrate is transported across the membrane by the ABC-transporter system followed by its step-wise oxidation to intermediates of general metabolism. Such plasmid-encoded sugar catabolic pathways integrated into the general metabolism of the cell have been previously reported for sucrose or for raffinose.

pAO1 is responsible for increased *A. nicotinovorans* cell-density when grown on xylose containing liquid media

The ability of *A. nicotinovorans* to assimilate various carbohydrates (such as L-Arabinose, D-Galactose, D-Glucose, L-Rhamnose, D-Ribose, D-xylose) has been previously reported (Kodama,

BCU IASI CENTRAL UNIVERSITY LIBRARY

Yamamoto et al., 1992). The standard method used follows the colonial growth of the bacterium on basal medium supplemented with carbohydrates and does not give any insights on the assimilation mechanisms. Although the pAO1 megaplasmid was identified at the time the two reports were published, its complete sequence was made available much later (Igloi and Brandsch, 2003) so none of the authors discussed the implications of the pAO1 megaplasmid in the mechanism of carbohydrates assimilation.

One simple question which one might ask is whether these assumed sugar-metabolism gene clusters are functional. Using the *A. nicotinovorans* pAO1+ and pAO1- strains a test was performed in an attempt to identify any differences regarding the ability of the two strains to use carbohydrates. 50 sugars and derivatives (heterosides, polyalcohols, uronic acids) were tested using the API 50CH kit (BioMerieux, Durham, USA) for fermentation and growing capabilities on various sugars. For the pAO1+ strain (the "wild type" strain originally isolated from soil), the results were in accordance with the previously reported findings of Kodama et al., 1992 and Keddie et al., 1986. In the case of the laboratory strain pAO1-, all of the results coincided with the pAO1+ strain except for D-xylose (Fig. 33, panel A).

BCU IASI CENTRAL UNIVERSITY LIBRARY

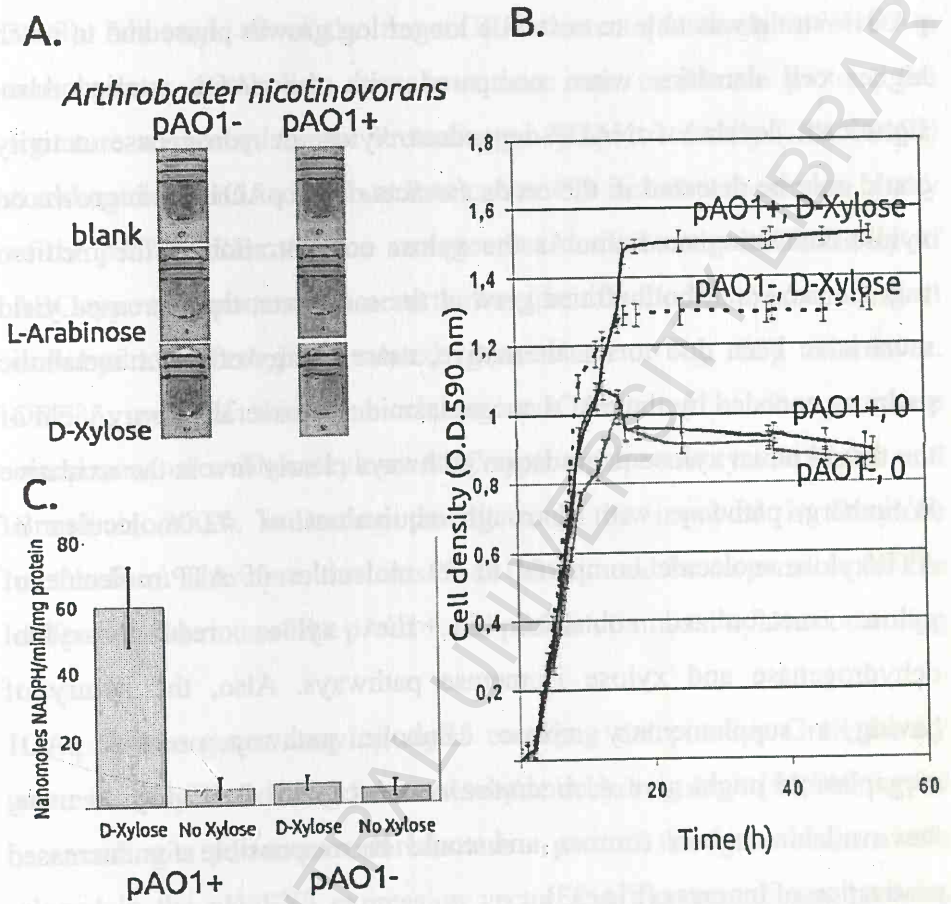


Fig. 33 – Differences regarding utilization of D-xylose between pAO1+ and pAO1- strains of *A. nicotinovorans* as revealed by the API 50CH test kit (A) and by growth curves on citrate media supplemented with D-xylose (B). C - xylose-dehydrogenase activity in the pAO1+ and pAO1- crude extracts. The error bars indicate standard deviations.

Although both strains were able to utilize D-xylose when grown on citrate medium supplemented with D-xylose (Fig. 33, panel B), the

BCUJASI CENTRAL UNIVERSITY LIBRARY

pAO1+ strain was able to sustain a longer log growth phase and to reach higher cell densities when compared with the pAO1- strain. Also, significant levels of NADP-dependent xylose-dehydrogenase activity could only be detected in the crude extracts of the pAO1 strain grown on xylose-containing medium. As the xylose concentration in the medium was the same and both strains grew at the same rate, the increased yield must have been due to an alternative, more energy-efficient metabolic pathway encoded by the pAO1 megaplasmid. The overall energy yield of the three known xylose degradation pathways clearly favors the oxidative Weimberg pathway with a rough equivalent of 42 molecules of ATP/xylose molecule compared to 29 molecules of ATP/molecule of xylose metabolized obtained in the xylose reductase-xylitol dehydrogenase and xylose isomerase pathways. Also, the luxury of having a supplementary xylose catabolic pathway on the pAO1 megaplasmid might give *A. nicotinovorans* increased flexibility for using the available carbon sources and could be responsible for increased production of biomass (Fig. 33).

Purification and characterization of ORF32, ORF39 and ORF40 proteins

Two main systems are known to be responsible for the transport of D-xylose across the bacterial membrane: the proton-symport system described in *E. coli* and various other bacteria (Singh and Mishra, 1995) and the MOS family of ABC-type transporters (Davidson, Dassa et al.,

BCU ASU CENTRAL UNIVERSITY LIBRARY

2008). Although all of the three ORF clusters putatively involved in carbohydrate metabolism contain at least one membrane protein, the signature sequences for the Proton/sugar symporter family (IPR018457) could not be identified by InterProScan or CD-Search web-servers in any of the pAO1 ORFs. A gene arrangement similar to the MOS family of ABC-type transporters was nevertheless identified in the third and largest carbohydrate-metabolism related gene cluster (Fig. 32). Moreover, ORF35, part of this gene cluster was predicted as the permease component of a ribose/xylose ABC-type transporter system (Igloi and Brandsch, 2003), indicating that it could be involved in D-xylose metabolism. Therefore, this cluster of ORFs could be responsible for the different behaviour of the pAO1+ and pAO1- strains on xylose containing media.

Three genes carrying *orf32* encoding the putative GntR family repressor, *orf39* – coding for an aldehyde dehydrogenase (ALDH), *orf40* – encoding a putative oxidoreductase - part of this gene cluster were cloned in the pH6EX3 expression vector and the recombinant proteins purified to more than 95% homogeneity by IMAC (Fig. 34).

BCU IASI / CENTRAL UNIVERSITY LIBRARY

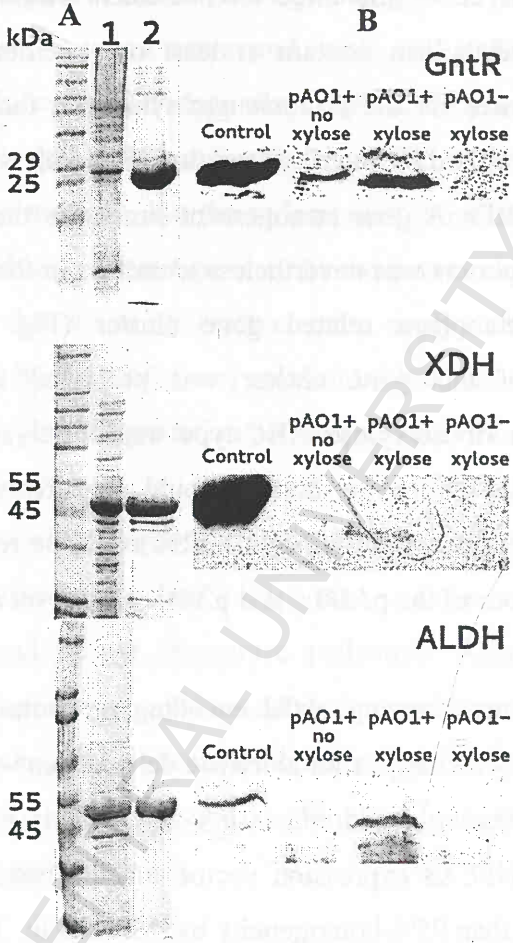


Fig. 34 – Purification and *in vivo* expression analysis of GntR, ALDH and XDH. A - SDS-PAGE: the proteins were purified to more than 95% homogeneity. 1) Total extracts from *E. coli* overexpressing the corresponding protein. 2) IMAC-purified His-tagged protein. B - Western blot detection of GntR, XDH, and ALDH respectively, in extracts of *A. nicotinovorans* pAO1+ grown on citrate medium (pAO1+, no xylose), *A. nicotinovorans* pAO1+ grown on citrate medium supplemented with 10 mM D-xylose (pAO1+, xylose) and *A. nicotinovorans* pAO1- grown on citrate medium supplemented with 10 mM D-xylose (pAO1-, xylose)

BCU IASI / CENTRAL UNIVERSITY LIBRARY

The ORF40 from A. *nicotinovorans* showed only low levels of sequence similarity with proteins with known function (30% sequence identity to 1,5-anhydro-D-fructose reductase from *Sinorhizobium morelense* (Kühn, Yu et al., 2006) and 20% sequence identity to D-xylose-NADP dehydrogenase from *Macaca fascicularis* (Arimitsu, Aoki et al., 1999). The presence of the highly conserved sequence AGKHVxCEKP for sugar dehydrogenases at position 119 in the ORF40 amino-acid sequence would further indicate that this enzyme is responsible for the first step in the putative oxidative pathway of xylose catabolism. Purified preparations of ORF40 showed a slight brown color at concentrations higher than 30 µg /µL. The protein had a relative molecular weight of 47 kDa and found to be homotetramer in solution. The Zn^{2+}, Fe^{2+} and Mn^{2+} content was analysed by atomic absorption spectroscopy and significant levels of Zn^{2+} were detected in the purified protein preparations. The metal/protein ratio corresponds to 2 Zn^{2+} atoms/monomer, a situation similar to other sugar dehydrogenases such as L-arabinitol 4-dehydrogenase from *Neurospora crassa* (Sullivan and Zhao, 2007) or manitol dehydrogenase from *Leuconostoc pseudomesenteroides* (Hahn, Kaup et al., 2003).

The purified recombinant ORF40 showed a $NADP^+$ dependent sugar dehydrogenase activity, with a strong preference for D-xylose (K_m = 15.2±0.23 mM (k_{cat} = 14.9± 0.5 s^{-1}, k_{cat}/K_m= 980 $s^{-1}M^{-1}$). At sugar concentrations of up to 50 mM of L-xylose, D-ribose, D-arabinose, L-arabinose, D-glucose, D-galactose or D-fructose the enzyme produced little NADPH and the specific activity towards these substrates remains

BCU IASI CENTRAL UNIVERSITY LIBRARY

below 5% of the activity recorded with D-xylose. The ORF40 was able to use both $NADP^+$ and NAD^+, but the analysis of xylose dehydrogenase activity over a range of co-enzyme concentration (0.1 μM – 100 mM) showed a strong preference for $NADP^+$ (for $NADP^+$ K_m= 0.32±0.03 mM, k_{cat}= 10.5± 0.5 s^{-1}, k_{cat}/K_m= 3.2 x 10^4 $s^{-1}M^{-1}$, vs. K_m= 5.03±0.2 mM, k_{cat}= 1.5± 0.2 s^{-1}, k_{cat}/K_m= 298 $s^{-1}M^{-1}$ for NAD^+). The ORF40 is thereby a $NADP^+/NAD^+$ D-xylose dehydrogenase (EC 1.1.1.175) and it will be referred as XDH hereafter. Similar strict specificity and K_m value for xylose were described by Yamanaka et al. (1977) for a xylose-dehydrogenase of *Arthrobacter* sp. no.588. Nevertheless, the differences regarding coenzyme specificity and native molecular weight (163 kDa for XDH of *A. nicotinovorans* and 62 kDa for the XDH of *Arthrobacter* sp. no.588) make us believe that these are two distinct enzymes.

The ALDH was initially predicted as a succinic-semialdehyde dehydrogenase (Igloi and Brandsch, 2003), showing 43% sequence identity with an $NAD(P)^+$ - dependent succinate semialdehyde dehydrogenase from *Escherichia coli* (Kurihara, Kato et al., 2010). Such an activity could not be identified with the 53 kDa monomeric His-tagged purified protein, but the enzyme was able to use both NAD^+ and $NADP^+$ to dehydrogenate various aldehydes such as formaldehyde, glyceraldehyde, butyraldehyde, glutaraldehyde and benzaldehyde. $NADP^+$ was preferred, the reaction being 20 times less eficient with NAD^+ when using butyraldehyde as substrate (Table 16). For active enzyme preparations, 5 mM mercaptoethanol was required in all buffers. When this component was omitted, the purified enzyme runs as a double

BCU IASI CENTRAL UNIVERSITY LIBRARY

band on SDS-PAGE, indicating the formation of an intra-molecular disulfide bond between 2 of the 5 Cys residues from the ALDH sequence. Sequence alignments with other members of the ALDH superfamily indicated that the Cys266 residue is highly conserved. This conserved Cys residue was shown to be responsible for the nucleophile attack on the aldehyde substrates as in the mechanism described by (Marchal and Branlant, 1999).

Table 16 – Kinetic parameters of ALDH determined from a hyperbolic Vmax model. Values correspond to the average of three replicates ± standard deviation

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
$NADP^+$	0.022±0.013	1.02±0.02	$4.6x10^4$
NAD^+	1.26±0.02	3.1±0.12	$2.4x10^3$
Formaldehyde[2]	4.2±1.1	0.38±0.03	9x10
Acetaldehyde[2]	2.5±0.6	0.75±0.11	$3x10^2$
Propionaldehyde[2]	1.5±0.1	1.2±0.11	$8x10^2$
Glyceraldehyde[2]	0.65±0.42	1.35±0.09	$2x10^3$
Butyraldehyde[2]	0.15±0.02	0.65±0.08	$4.3x10^3$
Glutaraldehyde[2]	0.11±0.03	3.92±0.06	$3.5x10^4$
Benzaldehyde[2]	2.1±0.2	4.52±0.12	$2.1x10^3$

[1] determined with 0.33 mM butyraldehyde

[2] determined with 0.1 mM $NADP^+$

BCU IAS / CENTRAL UNIVERSITY LIBRARY

Western-blots with *A. nicotinovorans* extracts

A. nicotinovorans pAO1+ and pAO1- cultures were grown for 48h on citrate medium supplemented with 10 mM D-xylose and the levels of GntR, ALDH, and XDH proteins were detected by western-blotting (Fig. 34, B). As expected from its predicted function as a repressor, the *gntR* gene was apparently constitutively expressed at low levels in pAO1+ strain regardless of the presence or absence of D-xylose. The *aldh* and *xdh* genes, which encode enzymes downstream the catabolic pathway, were expressed as expected only when D-xylose was present in the medium. No significant levels of expression could be detected when the cells were grown on various other sugars such as L-xylose, L-arabinose, D-galactose, D-tagatose and D-glucose.

Xylose degradation pathway in *Arthrobacter nicotinovorans* pAO1

The metabolic pathway of D-xylose in bacteria involves phosphorylation to xylose-5-P followed by the isomerization to D-xylulose-5-phosphate which is then channelled into the pentose phosphate pathway. The *xylA* chromosomal gene for xylose isomerase described in *Arthrobacter* strain B3728 as well as the putative similar genes present in all released genome sequences of *Arthrobacter* species: *A. arilaitensis* Re117, *A. phenantrenivorans*, *A. chlorophenolicus, A. sp. FB24* and *A.*

BCU JASSY CENTRAL UNIVERSITY LIBRARY

aurescens TC1 would indicate that in the genus *Arthrobacter* the metabolism of xylose is encoded by the chromosome.

As shown here, *A. nicotinovorans* pAO1- (lacking the pAO1 megaplasmid) was also able to grow on D-xylose presumably to these chromosomal *xyl* genes. The presence of the megaplasmid in *A. nicotinovorans* pAO1+ strain allowed the cells to grow to higher densities and, as no isomerase coding ORFs could be detected on the megaplasmid, an alternative pAO1 encoded pathway must be taken into account. The observed acidification of the growth medium would correspond well with the accumulation of xylonate reported by Yamanaka et al. (1977) and suggests a putative oxidative pathway similar to the Weimberg pentose pathway. In this pAO1 encoded pathway D-xylose may be taken up by the bacterial cells by the ABC-type transporter and then oxidised by the xylose-dehydrogenase XDH to D-xylonate, which may be further dehydrated by the ORF41 (GI:25169063) – a putative conserved protein belonging to the DHDPS-like family (cd00408) of proteins containing among others also a 5-keto-4-deoxy-glucarate dehydratase. The formed 2-keto glutarate semialdehyde could be further oxidised by the ORF39 protein (GI:25169061, aldehyde dehydrogenase) to 2-ketoglutarate. The later compound can then be integrated into the general metabolism of the cell.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

3.2. NICOTINE DERIVATIVES FROM *ARTHROBACTER NICOTINOVORANS* – NOVEL NEUROPROTECTIVE DRUGS?

It is estimated that 24 million people worldwide have Alzheimer's disease (AD) - characterized by a progressive deterioration of the cognitive and noncognitive functions.

In AD are affected some neurotransmitter systems (e.g. noradrenergic, histaminergic, serotonergic, dopaminergic). The most important feature of AD is the degeneration of the cholinergic system of the basal occipital lobe, such as neurons apoptosis within the basal nucleus of Meynert and degradation of cholinergic fibers in the cerebral cortex (especially in the temporal and frontal regions), hippocampus and amygdala. Additionally, in AD was observed a significant decline in acetylcholine level in the cortex and hippocampus and also, a decrease of acetylcholinetransferase activity (Lane et al., 2006).

The implication of nicotinic acetylcholine receptors (nAChR) in AD pathogenesis has led to the proposal of a new therapeutic approach. By using nAchR modulators to increase the availability of receptors for acetylcholine, the loss of forebrain cholinergic neurons can be overcome, and thereby the cognitive and non-cognitive functions can be improved. Nicotine is a well-known agonist of nAchR. Its effectiveness as a cognition-enhancing agent has been explained on one side by its ability to

BCU IASI CENTRAL UNIVERSITY LIBRARY

bind and modulate nAchRs and on the other side due to its anti-oxidant effects on the central nervous system at low concentrations.

In this context, the *Arthrobacter nicotinovorans* pAO1 strain, with its ability to metabolize nicotine, offers a wide range readily isolated of nicotine-derivatives with unexplored biotechnological potential. The nicotine catabolic pathway is placed on the 165 kB pAO1 megaplasmid and has been extensively characterized (Brandsch, 2006), all the intermediates from nicotine to the end-product a 4,5,4',5'-tetrahydroxy-3,3'-diazadiphenoquinone-(2,2')(nicotine-blue) being described. Moreover, all the enzymes involved in this degradation pathway have been purified and characterized.

3.2.1. *In-silico* identification of 6-hydroxy-L-nicotine as a novel neuroprotective drug

In order to identify metabolic derivatives with a potential use as neuroprotective drugs, the primary efforts were focused on the evaluation of the *Arthrobacter nicotinovorans* nicotine-intermediates potential to bind nAchRs by using a round of *in-silico* localized docking experiments in an attempt to select the best candidate for further *in-vitro* and *in-vivo* tests (Mihășan, Ștefan et al., 2013). This pilot computational study was aimed to *A. nicotinovorans* nicotine derivatives for the best drug candidate and to identify 6-hydroxy-L-nicotine (6HLN) as a compound with high potential in AD therapy.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

Receptors structure and ligand binding site

The nAChR is a large (290 kDa) glyco-protein, assembled from a ring of five homologous subunits, each subunit comprising of three domains: a small intracellular domain, a trans-membrane domain making the pore and a large N-terminal extracellular ligand- binding domain. There are five nicotine binding sites per nAChR molecule, each of them located in this last N-terminal domain, at the interface of two different subunits. Using such a large and highly variable molecule for *in vitro* and especially for *in silico* studies is rather difficult. For this, the acetylcholine binding protein AchBP (PDB ID 1UW6), a homolog of the ligand binding domain of nicotinic receptors and their family members is preferred. The AchBP is a homomeric water-soluble protein produced in the glia cells of *Lymnaea stagnalis* with pharmacological properties similar to the homomeric α7 subtype of the nAChRs (relatively weak affinity for acetylcholine and high affinity for nicotine). Because of the high sequence similarity (15% - 28% identity) to all ligand-gated ion channels, the crystal structure of AChBP is the established model for the extracellular domain of the pentameric AChR (Sixma and Smit, 2003) and also the perfect test subject for selecting nicotine derivatives with improved affinity towards nAChR.

In order to simplify the docking process and to make the screening more computational feasible, only one binding site was selected and used. A cube of aprox. 150 Å3 centered on Tyr143 from the subunit A of

BCU IASI CENTRAL UNIVERSITY LIBRARY

AchBP containing all the residues involved in ligand binding was the targeted region used for *in silico* docking experiments.

In-silico docking results

In order to consider a compound as a possible therapeutic agent for AD, it must have some nicotine-like effects, especially when it comes to nAChR binding and anti-oxidant properties. The potential of the *Arthrobacter nicotinovorans* nicotine intermediates to bind nAchRs was evaluated in round of *in silico* localized docking experiments. First, L-nicotine was docked into the binding site and compared with its experimentally observed orientation described by (Celie, van Rossum-Fikkert et al., 2004). The very good fit between the computationally obtained orientation of L-nicotine with the experimentally determined one (RMSD of 0.2 Å for 12 superimposed atoms of the ligand) indicate that the docking method used is reliable. The ligand with the best affinity for the AChBP binding site was found to be 6HLN - the first intermediate of the pathway.

The resulted theoretical binding pose of 6HLN was very similar to the one experimentally observed for nicotine with a RMSD of 0.19 Å for 12 atoms of the ligand (Fig. 35). Not only that this compound would fit into the binding pocket, but due to an extra H bond formed between the hydroxyl group of the 6HLN and Tyr185, the interaction energy calculated by AutoDockTools for 6HLN indicate that this compound would be more tightly bound compared to nicotine (theoretical binding

BCU JASI / CENTRAL UNIVERSITY LIBRARY

energy of -6.17 for nicotine compared to a binding energy of -6.66 for 6HLN).

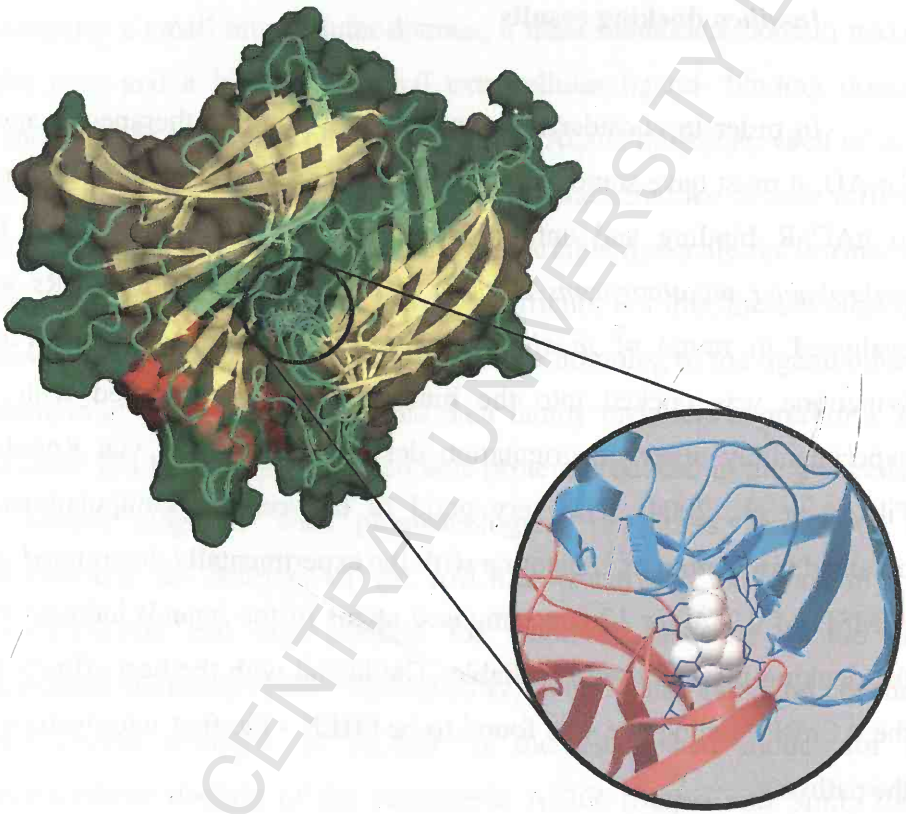


Fig. 35 – The AchBP (PDB ID 1UW6) structure and binding site. In the medallion, the best theoretical binding poses of 6HNic with the portion of AChBP molecule represented as cartoons, the residues described as involved in nicotine binding represented as sticks and the ligand as molecular surface.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

The anti-oxidant properties of 6HLN

The implications of the extra hydroxyl group in terms of 6HLN properties seem to go beyond the improved AchBP affinity. First, the OH makes this compound more soluble (Malphettes, Weber et al., 2005). Second, the anti-oxidant properties of 6HLN as calculated using the quantum chemical QSAR equation are improved compared with nicotine (for 6HLN the calculated IC50 = 43.06 compared to IC50 = 54.54 for nicotine).

Based on this theoretical data, from all the nicotine derivatives found in the *Arthrobacter nicotinovorans* catabolic pathway, 6HLN proved to be the strongest candidate for improving cognitive functions by means of modulating nAChR activity and by decreasing the oxidative stress in the brain. From a biotechnological point of view this is extremely convenient as the compound is formed directly from nicotine by a hydroxylation reaction catalyzed by the enzyme nicotine: acceptor oxido-reductase (hydroxylating) (E.C. 1.5.99.4, also known as nicotine dehydrogenase, NDH).

3.2.2. 6-hydroxy-L-nicotine from *Arthrobacter nicotinovorans* sustain spatial memory formation by decreasing brain oxidative stress in rats

In order to verify if 6HLN can be used as potential neuroprotective drug, a study was designed to determine whether this compound affects

BCU IASI / CENTRAL UNIVERSITY LIBRARY

spatial memory formation in Y-maze and radial arm maze tasks (Hriţcu, Ştefan et al., 2013). Furthermore, it is interesting to know if 6HLN exposure would result in an imbalance in the oxidative stress levels of temporal lobe, the most vulnerable cortical area to oxidative stress effects, by measuring the extent of some lipid peroxidation products (malondialdehyde - MDA) and defense enzymes (superoxide-dismutase - SOD and glutathione peroxidase - GPX).

Effects of chronic 6HLN administration on spatial memory

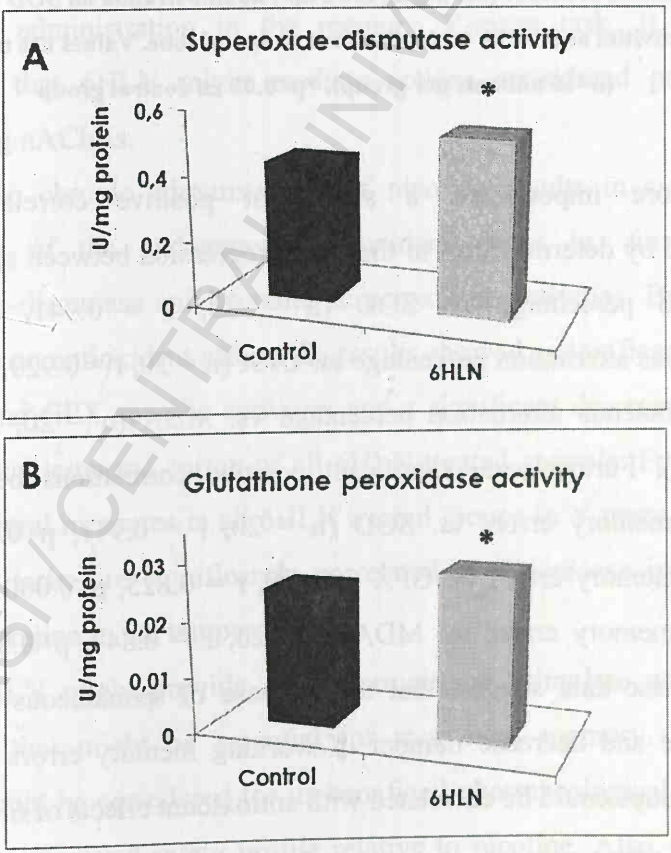
In Y-maze task, it was observed a significant improvement of spatial memory in 6HLN-treated group ($F(1.8) = 62.4$, $p<0.0001$), indicated by an increase of spontaneous alternation percentage compared to control group, suggesting effects on short term memory. This effect could be attributed to increased motor activity, because 6HLN induced an increase in locomotor activity ($F(1.8) = 81.7$, $p<0.0001$). A significant improvement of working memory in all 6HLN treated rats, during 7 days training in radial arm maze task was found. Repeated measures ANOVA revealed a significant group difference for: working memory errors ($F(1.42) = 13.41$, $p<0.001$; entries to repeat ($0.7<p<0.84$); time taken to consume all five baits ($F(1.56) = 10.30$, $p<0.002$).

Long-term memory was unaffected during 7 days training of 6HLN treated rats in radial arm-maze task, no significant main effects or significant interactions for reference memory errors were observed ($0.06<p<0.79$).

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

Effects of chronic 6HLN administration on SOD and GPX activities and MDA level in rat temporal lobe

6HLN significantly increased SOD ($F(1.14)04.48$, $p<0.05$) and GPX ($F(1.14)=7.9$, $p<0.05$) specific activities and significantly decreased MDA level ($F(1.14)=2.92$, $p<0.01$) in rat temporal lobe compared to the control group, implying that 6HLN may have induced a decrease in neuronal oxidative stress – Fig. 36.



BCU IAȘI / CENTRAL UNIVERSITY LIBRARY

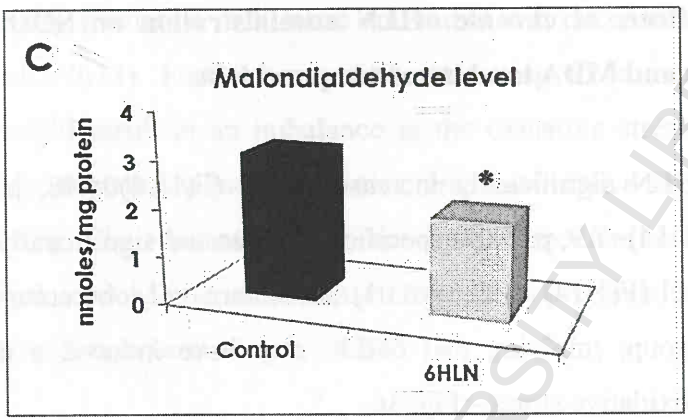


Fig. 36 – Effects of 6HLN (0.3 mg/kg b.w., i.p.) administration on SOD (a), GPX (b) specific activities and MDA (c) levels in rat temporal lobe. Values are mean ± SEM (n=10 animals per group), *p<0.05 *vs.* control group

More importantly, a significant positive correlations was evidenced by determination of the linear regression between spontaneous alternation percentage *vs.* SOD ($n = 20$, $r = 0.941$, $p<0.0001$), spontaneous alternation percentage *vs.* GPX ($n = 20$, $r = 0.929$, $p<0.0001$) and spontaneous alternation percentage vs. MDA ($n = 20$, $r = 0.951$, $p<0.0001$). Furthermore, significant positive correlations between the working memory errors *vs.* SOD ($n = 20$, $r = 0.941$, $p<0.0001$), the working memory errors *vs.* GPX ($n = 20$, $r = 0.825$, $p<0.0001$), and the working memory errors *vs.* MDA ($n = 20$, $r = 0.849$, $p<0.0001$) were found. These data suggest that the increase of spontaneous alternation percentage and decrease number of working memory errors in 6HLN-treated groups could be correlated with antioxidant effects of 6HLN.

BCU MS / CENTRAL UNIVERSITY LIBRARY

6HLN has positive effects on spatial memory, especially on short term memory and working memory, and also on antioxidant status in rat's brain. There are no studies performed to date to demonstrate the effects of 6HLN, derived from nicotine catabolism in *A. nicotinovorans*, on spatial memory and oxidative stress.

The results showed that 6HLN improves spatial memory with the same effectiveness as nicotine, especially on short-term memory and on working memory within Y-maze and radial arm-maze tasks. In contrast with nicotine, the animals displayed increased locomotor activity as result of 6HLN administration in the memory Y-maze task. It might be suggested that 6HLN might mediate actions considered positive by stimulating nAChRs.

Also chronic administration of nicotine results in significantly decreasing of the endogenous antioxidant status in, for example, superoxide-dismutase and glutathione peroxidase activities. By contrast, 6HLN has an antioxidant effect: the results showed a significant increase of SOD and GPX specific activities and a significant decrease of MDA levels in the temporal cortex of all 6HLN-treated animals. Furthermore, the behavioral measures in all 6HLN treated groups in Y-maze and radial arm-maze tasks are significantly correlated to a decrease in oxidative stress generation in the temporal lobe.

6HLN might provide an opportunity to stimulate neurological processes that might be essential for short-term memory. Moreover, 6HLN should be considered for its beneficial pharmacological properties and for its improved safety profile relative to nicotine. Also, the results

BOUIASI CENTRAL UNIVERSITY LIBRARY

extend previous researches on the nicotine metabolites effects on memory processes. Nevertheless, several open questions regarding the feasibility of using 6HLN as an alternative AD therapy still remain unanswered. One question is related to the ability of 6HLN to cross the blood brain and its systemic toxicity in relation with nicotine. A second question takes into account the various nAchRs pentameric subtypes and concern the specificity of this compound in regard to nAchR subunits and the relevance of this specificity for Alzheimer's disease.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

CONCLUSIONS

Applied microbiology is equally about recombinant DNA, cloning and genetics as well as about producing antibiotics and new drugs; it is also about solving current problems of the humankind as the need for providing food and a healthy environment.

The microorganisms can be used for increasing agricultural food production in *green* conditions. Identification of different mechanisms involved in plant-rhizosphere microorganism interaction opened new possibilities to design strategies for improving crop yields. In the present book were described many beneficial effects of plant growth promoting rhizobacteria, from enhancing growth and physiological processes to yield increase. All the scientific data offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements with biofertilizers - reliable environmental friendly component in the management of sustainable agricultural systems. Nevertheless the utilization of rhizobacteria in agriculture depends on our knowledge about bacteria - plant interaction and our ability to maintain, manipulate and modify beneficial bacterial populations under field conditions.

One of the main current medical problems is the widespread emergence of acquired bacterial resistance to antibiotics in such a way that the world is facing today a serious threat to global public health in the form of not only epidemics, but also pandemics of antibiotic resistance. Identification of new antibacterial agents such as plant extracts or noble

BCU IASI - CENTRAL UNIVERSITY LIBRARY

metale nanoparticles can offer a reliable alternative to antibiotics in the fight against multidrug resistant bacteria.

Due to their versatile metabolic properties, many bacteria such as *Arthrobacter nicotinovorans* are used as important sources for valuable compounds. In this line, some new biotechnological applications of metabolic derivatives from *A. nicotinovorans* cells such as enzymes needed for efficient use of lignocellulose and nicotine metabolites as novel neuroprotective drugs were presented here. In order to cover the increasing humankind demand for fuels, lignocellulose provides a promising alternative due to its high abundance and renewable character. The metabolic pathways encoded by the pAO1 megaplasmid of *A. nicotinovorans* can offer enzymes with a broader optimal pH and temperature and better solvent stability needed for efficient use of lignocellulose and its derivative D-xylose.

Degradation of nicotine in *A. nicotinovorans* is also based on the catabolic machinery encoded by pAO1 megaplasmid. Numerous studies indicated nicotine as an alkaloid with important impact on brain functions. In this context, the *A. nicotinovorans* pAO1 strain, with its ability to metabolize nicotine, offers a wide range readily isolated of nicotine-derivatives with unexplored biotechnological potential. Therefore it has become increasingly important to obtain more information on the biological effects of nicotine metabolites derived from mammalian or bacterial cells, in order to possibly manage neurological abnormalities in some neurodegenerative afflictions. The data presented so far showed that 6-hydroxy-L-nicotine (a nicotine metabolite derived from nicotine

BCU IASI / CENTRAL UNIVERSITY LIBRARY

catabolism in *A. nicotinovorans*) affects spatial memory formation and brain antioxidant with good potential to be used as a possible drug for Alzheimer disease.

The use of microorganisms in agriculture, chemical industry and medicine will improve the standard of our everyday life from better medical treatments to increase crop yields and will sustain growth and development in countries throughout the world for several decades to come.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

REFERENCES

ORIGINAL PAPERS

ISI JOURNALS

1. Hritcu, L., **Stefan, M.**, Brandsch, R., Mihasan, M., 2012 - *6-hydroxy-L-nicotine from Arthrobacter nicotinovorans sustain spatial memory formation by decreasing brain oxidative stress in rats*, Journal of Physiology and Biochemistry, 69(1):25-34, DOI: 10.1007/s13105-012-0184-9.
(http://link.springer.com/article/10.1007%2Fs13105-012-0184-9)
With kind permission from Springer Science+Business Media: Journal of Physiology and Biochemistry, 6-hydroxy-L-nicotine from *Arthrobacter nicotinovorans* sustain spatial memory formation by decreasing brain oxidative stress in rats, 69(1), 2012, 25-34, Hritcu, L., Stefan, M., Brandsch, R., Mihasan, M.
2. Mihasan, M., **Stefan, M.**, Hritcu, L., Brandsch, R., 2013 - *Evidence of a plasmid-encoded oxidative xylose catabolic pathway in Arthrobacter nicotinovorans pAO1*, Research in Microbiology, 164: 22-30, DOI: 10.1016/j.resmic.2012.10.003.
(http://www.sciencedirect.com/science/article/pii/S0923250812001374)
Reproduced from *Mihasan, M., Stefan, M., Hritcu, L., Brandsch, R. Evidence of a plasmid-encoded oxidative xylose catabolic pathway in Arthrobacter nicotinovorans pAO1. Research in Microbiology, 2013, 164: 22-30.* Copyright © *2013* Elsevier Masson SAS. All rights reserved.

BCU "ASACHI" CENTRAL UNIVERSITY LIBRARY

3. Mihăşan, M., Luminita, C., Neagu, E., **Stefan, M.**, Hritcu, L., 2013 - *In-silico identification of 6-hydroxy-L-nicotine as a novel neuroprotective drug*, Romanian Biotechnological Letters, vol. 18, no.3,8333-8340.
(http://www.rombio.eu/vol18nr3/14%20Mihasan.pdf)
4. Noumedem, J.A.K., Mihasan, M., Lacmata, S.T., **Ştefan, M.**, Kuiate, J.R., Kuete, V., 2013 - *Antibacterial activities of the methanol extracts of ten Cameroonian vegetables against Gram-negative multidrug-resistant bacteria*, BMC Complementary and Alternative Medicine, 13:26, 1-9, DOI:10.1186/1472-6882-13-26.
(http://www.biomedcentral.com/1472-6882/13/26)
5. Noumedem, J.A.K., Mihasan, M., Kuiate, J.R., **Ştefan, M.**, Cojocaru, D., Dzoyem, J.P., Kuete, V., 2013 - *In Vitro antibacterial and antibiotic-potentiation activities of four edible plants against multidrug-resistant Gram-negative species*, BMC Complementary and Alternative Medicine, 13, Article Number – 190, DOI:10.1186/1472-6882-13-190.
(http://www.biomedcentral.com/1472-6882/13/190)
6. **Ştefan, M.**, Melnig, V., Pricop, D., Neagu, A., Mihăşan, M., Tartau, L., Hritcu, L., 2013 - *Attenuated effects of chitosan-capped gold nanoparticles on LPS-induced toxicity in laboratory rats*, Materials Science and Engineering: C Materials for Biological Applications, 33:550-556, DOI: 10.1016/j.msec.2012.09.031.
(http://www.sciencedirect.com/science/article/pii/S0928493112004638)
Reprinted from Materials Science and Engineering: C Materials for Biological Applications, 33, Stefan, M., Melnig, V., Pricop, D., Neagu, A., Mihasan, M., Tartau, L., Hritcu, L., Attenuated effects of chitosan-capped

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

gold nanoparticles on LPS-induced toxicity in laboratory rats, 550-556, Copyright (2013), with permission from Elsevier.

7. **Ştefan, M.**, Munteanu, N., Stoleru, V., Mihasan, M., Hritcu, L., 2013 - *Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean (Phaseolus coccineus L.)*, Scientia Horticulturae, 151: 22-29, DOI: 10.1016/j.scienta.2012.12.006.

(http://www.sciencedirect.com/science/article/pii/S0304423812005833) Reprinted from Scientia Horticulturae, 151, Stefan, M., Munteanu, N., Stoleru, V., Mihasan, M., Hritcu, L., Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean (*Phaseolus coccineus* L.), 22-29, Copyright (2013), with permission from Elsevier.

8. **Ştefan, M.**, Zamfirache, M.M., Padurariu, C., Trută, E., Gostin, I., 2013 - *The composition and antibacterial activity of essential oils in three Ocimum species growing in Romania*, Central European Journal of Biology, 8(6), 600-608, DOI:10.2478/s11535-013-0171-8.

(http://link.springer.com/article/10.2478%2Fs11535-013-0171-8) With kind permission from Springer Science+Business Media: Central European Journal of Biology, The composition and antibacterial activity of essential oils in three *Ocimum* species growing in Romania, 8(6), 2013, 600-608, Stefan, M., Zamfirache, M.M., Padurariu, C., Trută, E., Gostin, I.

9. **Ştefan, M.**, Munteanu, N., Stoleru, V., Mihasan, M., 2013 - *Effects of inoculation with plant growth promoting rhizobacteria on photosynthesis, antioxidant status and yield of runner bean*, Romanian Biotechnological Letters, Vol. 18, No.2, 8132-8143.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

(http://www.rombio.eu/vol18nr2/8%20Marius%20Stefan-Neculai.pdf)

10. **Ştefan, M.**, Hritcu, L., Mihasan, M., Pricop, D., Gostin, I., Olariu, R.I., Dunca, S., Melnig, V, 2011 - *Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media*, Journal of Materials Science: Materials in Medicine, 22: 789-796, DOI: 10.1007/s10856-011-4281-z.

(http://link.springer.com/article/10.1007%2Fs10856-011-4281-z)

With kind permission from Springer Science+Business Media: Journal of Materials Science: Materials in Medicine, Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media, 22, 2011, 789-796, Stefan, M., Hritcu, L., Mihasan, M., Pricop, D., Gostin, I., Olariu, R.I., Dunca, S., Melnig, V.

11. **Ştefan, M.,** Dunca, S., Olteanu, Z., Oprica, L., Ungureanu, E., Hritcu, L., Mihasan, M., Cojocaru, D., 2010 - *Soybean (Glycine max [L] Merr.) inoculation with Bacillus pumilus Rs3 promotes plant growth and increases seed protein yield: relevance for environmentally-friendly agricultural applications,* Carpathian Journal of Earth and Environmental Sciences, Vol. 5, No. 1, 131-138.

(http://www.ubm.ro/sites/CJEES/viewTopic.php?topicId=83)

12. Zamfirache, M.M., Burzo, I., Gostin, I., **Stefan**, M., Padurariu, C., Olteanu, Z., Badea, M.L., Lamban, C., Truta, E., Ivanescu, L., Cojocaru, D., 2010 - *Micromorphology of secretory trichomes and chemical composition of volatile oils in Perovskia atriplicifolia benth. from north-eastern Romania.* Carpathian Journal of Earth and Environmental Sciences, Vol. 6, No. 2, 261-268.

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

(http://www.ubm.ro/sites/CJEES/viewTopic.php?topicId=179)

INTERNATIONAL DATABASES JOURNALS

1. Achitei, E., **Ştefan, M.**, Mihasan, M., Hritcu, L., Dunca, S., 2010 - *Siderophores and indole-3-acetic acid production by bacterial strains isolated from soybean rhizosphere*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XI, Fasc. 4, p. 59-65.
2. Apostu, A., Petriman, N., Trăsnea, I., Mihasan, M., Dunca, S., **Ştefan, M.**, 2010 - *Rhizobacterial strains with phosphorus solubilizing capabilities*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XI, Fasc. 4, p. 67-74.
3. Cazacu, A., Bindar, D., Tartau, L., Hritcu, L., **Ştefan, M.**, Nita, L., Ionescu, C., Nica, V., Rusu, G., Dobromir, M., Melnig, V., 2011 - *Effect on nerve structures of functionalized gold chitosan nanoparticles obtained by one pot synthesis*, Analele Ştiintifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XII, p. 45-50.
4. Dunca, S., **Ştefan, M.**, Tănase, C., Cojocariu, A., 2008 - *Evaluation of the rhizospheric microbiota from soils degraded by mining activities*, Natura Montenegrina, 7(3), p. 17-26.
5. Hriţcu, L., **Ştefan, M.**, Mihăşan, M., Brandsch, R., 2010 - *6-hydroxy-L-nicotine from Arthrobacter nicotinovorans facilitate spatial memory formation in rats*, Analele Ştiintifice ale Universităţii Alexandru Ioan

BCU IAŞI CENTRAL UNIVERSITY LIBRARY

Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XI, Fasc. 4, p. 207-211.

6. Mihalache, G., Munteanu, N., Dunca, S., Mihasan, M., **Ştefan, M.**, 2012 - *Phosphate-solubilising rhizobacteria associated with Phaseolus coccineus L. rhizosphere*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM XIII, Fasc. 3, p. 65-70.
7. Mihăşan, M., **Ştefan, M.**, Artenie, V., 2012 - *Experimental evidence of a xylose-catabolic pathway on the pAO1 megaplasmid of Arthrobacter nicotinovorans*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, Vol 12, No 1, p. 19-26.
8. Mihăşan, M., **Ştefan, M.**, Artenie, V., Brandsch, R., 2011 - *Cloning and purification of a repressor protein from Arthrobacter nicotinovorans PAO1*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XII, p. 21-25.
9. Mihăşan, M., **Ştefan, M.**, 2008 - *The evolution of total soluble proteins content during the germination of Glycine max L. beans under the influence of some rhizobacterial strains*, Lucrări ştiinţifice, seria Agronomie, Iaşi, vol. 51 (2), p. 7-13.
10. **Ştefan, M.**, Munteanu, N., Mihasan, M., 2013 - *Application of plant growth-promoting rhizobacteria to runner bean increases seed carbohydrate and protein yield*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM XIV, Fasc.1, p. 29-36.

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

11. **Ştefan, M.**, Munteanu, N., Dunca, S., 2012 - *Plant-microbial interactions in the rhizosphere – strategies for plant growth-promotion*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM XIII, Fasc.1, p. 87-96.

12. **Ştefan, M.**, Hritcu, L., Obreja, L., Melnig, V., 2010 - *Synthesis and antibacterial effect of silver nanoparticles with different sizes*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM XI, Fasc.1, p. 99-106.

13. **Ştefan, M.**, Mihasan, M., Raus, L., Topa, D., Dunca, S., Hritcu, L., 2009 - *Rhizosphere bacteria help protein accumulation in soybean seeds*. Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM X, Fasc. 2, p. 23-29.

14. **Ştefan, M.**, Mihăşan, M., Dunca, S., 2008 - *Plant growth promoting rhizobacteria can inhibit the in vitro germination of Glycine max L. seeds*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM IX, Fasc. 3, p. 105-110.

15. **Ştefan, M.**, Mihasan, M., Raus, L., Topa, D., Dunca, S., 2008 - *Agriculture applications of some rhizobacterial strains isolated from Moldavian plaine cambic - chernozemic soils*, Lucrări ştiinţifice, seria Agronomie, Iaşi, vol. 51 (1), p. 191-196.

16. **Ştefan, M.**, Ungureanu, E., Oprică, L., Dunca, S., 2007 - *The effect of some rhizobacterian strains on soluble proteins content in soybeans*

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

(Glycine max L. Merr.), Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM VIII, p. 23-26.

17. **Ştefan, M.**, Ungureanu, E., Oprică, L., Dunca, S., 2007 - *The impact of using some rhizobacterial strains as biofertilizers on the total content of soluble proteins in corn caryopses (Zea mays)*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM VIII, p. 85-88.
18. **Ştefan, M.**, Zenovia, O., Oprică, L., Dunca, S., Vîntu, S., 2006 - *Impact of some PGPR on Zea mays "in vitro" development as environmental biotechnology application*, 6th International Scientific Conference Anniverssary Issue, Modern Management of Mine Producing, Geology and Environmental Protection, Vol. II, p. 51-55.
19. **Ştefan, M.**, Ailiesei, O., Ungureanu, E., Dunca, S., Nimiţan, E., Artenie, V., 2005 - *Study of a microbial inoculum on several biochemical indices in sunflower (Helianthus anuus L.)*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM V, p. 11-14.
20. **Ştefan, M.**, Olteanu, Z., Oprică, L., Dunca, S., 2005 - *Impact of rhizobacteria in some enzimatic processes in maize plants (Zea mays L.) in vitro*, Lucrări ştiinţifice, Seria Agronomie, vol. 48.
21. **Ştefan, M.**, Ailiesei, O., Ungureanu, E., Maniu, C., Dunca, S., Răuş, L., 2005 - *Environmental and agriculture applications of PGPR's strains isolated from Zea mays L. rhizosphere*, 5th International Scientific Conference Anniverssary Issue, Modern Management of

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

Mine Producing, Geology and Environmental Protection, 13-17 Iunie, Bulgaria, p. 281-286.

22. **Ştefan, M.**, Maniu, C., Neacşu, I., Dunca, S., 2005 - *Influenţa unor tulpini bacteriene rizosferice asupra preluării de către plantele de porumb a unor elemente minerale in vitro*, Analele Societăţii Naţionale de Biologie Celulară,Vol. X, p. 507-511.
23. **Ştefan, M.**, Ailiesei, O., Nimiţan, E., Dunca, S., Zamfirache, M.M., Ungureanu, E., 2005 - *The effect of plant-grow-promoting rhizobacteria on some physiological processes occuring during the "in vitro" germination of maize (Zea mays L.)*, Natura Montenegrina, No. 3, p. 154-157.
24. **Ştefan, M.**, Ailiesei, O., Nimiţan, E., Dunca, S., Ungureanu, E., 2004 - *The effect of several rhizospheric microorganisms on the content of starch in maize (Zea mays L.) caryopses during the* in vitro *germination*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM V, p. 150-153.
25. **Ştefan, M.**, Ailiesei, O., Ungureanu, E., Zamfirache, M.M., Nimiţan, E., Dunca, S., 2004 - *The effect of several rhizospheral microorganisms on a number of energy – generating processes occurring during the "in vitro" germination of maize (Zea mays L.) caryopses,* Lucrările ştiinţifice (anul XLVII), vol. I (47), Seria Horticultură, p. 1265-1268.
26. **Ştefan, M.**, Ailiesei, O., Nimiţan, E., Dunca, S., 2003 - *Aspects of the rhizosphere effect in a Zea mays L. genotype,* Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară, TOM IV, p. 17-20.

BCU IAŞI CENTRAL UNIVERSITY LIBRARY

27. Zamfirache, M.M., Burzo, I., Gostin, I., Olteanu, Z., **Ştefan, M.**, Badea, M.L., Padurariu, C., Gales, R.C., Adumitresei, L., Lamban, C., Truţă, E., Stănescu, I., 2009 - *Glandular trichomes and essential oil constituents of Perovskia atriplicifolia benth.*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM X, Fasc. 3, p. 73-80.

28. Zamfirache, M.M., Burzo, I., Olteanu, Z., Dunca, S., Surdu, Ş., Truţă, E., **Ştefan, M.**, Roşu, C.M., 2008 - *Research regarding the volatile oils composition for Ocimum basilicum L. and their possible phytotherapeutic effects*, Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi (Serie Nouă). Secţiunea II-a Genetică şi Biologie Moleculară, TOM IX, Fasc. 2, p. 35-40.

29. Zamfirache, M.M., Toma, C., Duca, M., Dunca, S., Olteanu, Z., **Ştefan, M.**, Galeş, R., Pădurariu, C., 2008 - *A comparative study regarding the morphology and anatomy of the vegetative apparatus in two Ocimum basilicum L. breeds*, Analele Ştiinţifice ale Universităţii Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Biologie vegetală, TOM LIV, Fasc. 2, p. 38-47.

30. Zamfirache, M.M., Burzo, I., Olteanu, Z., Toma, C., Truta, E., Ivanescu, L., Dunca, S., Surdu, S., Rosu, C., **Stefan, M.**, 2008 - *Researches concerning the volatile oils produced by some species of aromatic plants cultivated in the northern area of Romania and their possible phytotherapeutic effects*, Natura Montenegrina, 7(2), p. 593-604.

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

OTHER REFERENCES

1. Achitei, E., M. Stefan, M. Mihasan, L. Hritcu and S. Dunca, 2010 - *Siderophores and indole-3-acetic acid production by bacterial strains isolated from soybean rhizosphere*. Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iasi (Serie Nouă). Secţiunea II. Genetică si Biologie Moleculară **XI**(4): 59-65.
2. Ahmad, F., I. Ahmad and M. S. Khan, 2005 - *Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of Tryptophan*. Turk J Biol **29**: 29–34.
3. Ahmad, F., I. Ahmad and M. S. Khan, 2008 - *Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities*. Microbiol. Res. **163**(2): 173-181.
4. Akubugwo, I. E., N. A. Obasi, G. C. Chinyere and A. E. Ugbogu, 2007 - *Nutritional and chemical value of Amaranthus hybridus L. leaves from Afikpo Nigeria*. Afr J Biotechnol **6**(24): 2833–2839.
5. Al-Zuhair, H., A. A. Abd El-Fattah and H. A. Abd El Latif, 1997 - *Efficacy of Simvastatin and pumpkin-seed oil in the management of dietary-induced hypercholesterolemia*. Pharmacological Research **35**(5): 403-408.
6. Albone, K., P. Gaskin, J. MacMillan and V. Sponsel, 1984 - *Identification and localization of gibberellins in maturing seeds of the cucurbit Sechium edule, and a comparison between this cucurbit and the legume Phaseolus coccineus*. Planta **162**(6): 560-565.

BCU IASI CENTRAL UNIVERSITY LIBRARY

7. Allen, R. D., 1995 - *Dissection of Oxidative Stress Tolerance Using Transgenic Plants*. Plant Physiology **107**(4): 1049-1054.
8. Amarowicz, R., G. A. Dykes and R. B. Pegg, 2008 - *Antibacterial activity of tannin constituents from Phaseolus vulgaris, Fagoypyrum esculentum, Corylus avellana and Juglans nigra*. Fitoterapia **79**(3): 217-219.
9. Amir, H. G., Z. H. Shamsuddin, M. S. Halimi, M. F. Ramlan and M. Marziah, 2001 - *Effects of Azospirillum inoculation on N2 fixation and growth of oil palm plantlets at nursery stage*. J. Oil Palm Res. **13** (1): 42–49.
10. Antoun, H., C. J. Beauchamp, N. Goussard, R. Chabot and R. Lalande, 1998 - *Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (Raphanus sativus L.)*. Plant and Soil **204**(1): 57-67.
11. Apostu, A., N. Petriman, I. Trăsnea, M. Mihasan, S. Dunca and S. Stefan, 2010 - *Rhizobacterial strains with phosphorus solubilizing capabilities*. Analele Stiintifice ale Universitatii Alexandru Ioan Cuza din Iasi (Serie Nouă). Secţiunea II. Genetică si Biologie Moleculară **XI**(4): 67-74.
12. Arimitsu, E., S. Aoki, S. Ishikura, K. Nakanishi, K. Matsuura and A. Hara, 1999 - *Cloning and sequencing of the cDNA species for mammalian dimeric dihydrodiol dehydrogenases*. Biochem. J. **342**(3): 721-728.
13. Baakza, A., A. K. Vala, B. P. Dave and H. C. Dube, 2004 - *A comparative study of siderophore production by fungi from marine and terrestrial habitats*. Journal of Experimental Marine Biology and Ecology **311**(1): 1-9.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

14. Badri, D. V., T. L. Weir, D. van der Lelie and J. M. Vivanco, 2009 - *Rhizosphere chemical dialogues: plant–microbe interactions.* Current Opinion in Biotechnology **20**(6): 642-650.
15. Bai, Y., X. Zhou and D. L. Smith, 2003 - *Enhanced soybean plant growth resulting from coinoculation of Bacillus strains with Bradyrhizobium japonicum.* Crop Sci. **43**: 1774-1781.
16. Bai, Y., X. Zhou and D. L. Smith, 2003 - *Enhanced Soybean Plant Growth Resulting from Coinoculation of Bacillus Strains with Bradyrhizobium japonicum.* Crop Sci **43**(5): 1774-1781.
17. Bar-Ness, E., Y. Chen, Y. Hadar, H. Marschner and V. Römheld, 1991 - *Siderophores of Pseudomonas putida as an iron source for dicot and monocot plants.* Plant and Soil **130**(1): 231-241.
18. Bar-Ness, E., Y. Hadar, Y. Chen, V. Römheld and H. Marschner, 1992 - *Short-Term Effects of Rhizosphere Microorganisms on Fe Uptake from Microbial Siderophores by Maize and Oat.* Plant Physiol. **100**(1): 451–456.
19. Bardiya, M. C., A. C. Gaur and I. a. s. o. m. d. l. g. r. phosphate, 1974 - *Isolation and screening of microorganisms dissolving low grade rock phosphate.* Folia Microbiol **19**: 386–389.
20. Barkat, M. and F. Kadri, 2011 - *Impact de deux modes de cuisson sur la teneur en polyphénols solubles de six légumes.* Revue de genie industriel **6**: 41–45.
21. Baset Mia, M. A., Z. H. Shamsuddin, Z. Wahab and M. Marziah, 2010 - *Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (Musa spp. cv. 'Berangan').* Sci. Hortic. **126**(2): 80-87.

BCU JSC CENTRAL UNIVERSITY LIBRARY

22. Bashan, I., 1998 - *Inoculants of plant growth-promoting bacteria for use in agriculture.* Biotechnol. Adv. **16**: 729-770.

23. Bashan, Y., G. Holguin and R. Lifshitz, 1993 - *Isolation and Characterization of Plant Growth-Promoting Rhizobacteria.* Methods in Plant Molecular Biology and Biotechnology. B. R. G. J. E. Thompson. Washington, D.C., USA CRC Press, Inc.: 384.

24. Belimov, A. A., A. P. Kojemiakov and C. V. Chuvarliyeva, 1995 - *Interaction between barley and mixed cultures of nitrogen fixing and phosphate-solubilizing bacteria.* Plant and Soil **173**(1): 29-37.

25. Benedec, D., I. Oniga, R. Oprean and M. Tamas, 2009 - *Chemical composition of the essential oils of Ocimum basilicum L. cultivated In Romania.* Farmacia **57**(5): 625-629.

26. Benizri, E., E. Baudoin and A. Guckert, 2001 - *Root Colonization by Inoculated Plant Growth-Promoting Rhizobacteria.* Biocontrol Science and Technology **11**(5): 557-574.

27. Bhattacharyya, P. and D. Jha, 2012 - *Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture.* World Journal of Microbiology and Biotechnology: 1-24.

28. Boca, S. C., M. Potara, F. Toderas, O. Stephan, P. L. Baldeck and S. Astilean, 2011 - *Uptake and biological effects of chitosan-capped gold nanoparticles on Chinese Hamster Ovary cells.* Materials Science and Engineering: C **31**(2): 184-189.

29. Bozin, B., N. Mimica-Dukic, N. Simin and G. Anackov, 2006 - *Characterization of the Volatile Composition of Essential Oils of Some Lamiaceae Spices and the Antimicrobial and Antioxidant*

BCU IASI / OF TRANSILVANIA UNIVERSITY LIBRARY

Activities of the Entire Oils. Journal of Agricultural and Food Chemistry **54**(5): 1822-1828.

30. Braga, L. C., A. A. M. Leite, K. G. S. Xavier, J. A. Takahashi, M. P. Bemquerer, E. Chartone-Souza and A. M. A. Nascimento, 2005 - *Synergic interaction between pomegranate extract and antibiotics against Staphylococcus aureus*. Can J Microbiol **51**(17): 541–547.
31. Brandi, G., G. Amagliani, G. F. Schiavano, M. De Santi and M. Sisti, 2006 - *Activity of Brassica oleracea Leaf Juice on Foodborne Pathogenic Bacteria*. Journal of Food Protection **69**(9): 2274-2279.
32. Brandsch, R., 2006 - *Microbiology and biochemistry of nicotine degradation*. Appl Microbiol Biotechnol(69): 493-498.
33. Brandsch, R. and K. Decker, 1984 - *Isolation and partial characterization of plasmid DNA from Arthrobacter oxidans*. Arch Microbiol **138**: 15-17.
34. Braydich-Stolle, L., S. Hussain, J. J. Schlager and M. C. Hofmann, 2005 - *In vitro cytotoxicity of nanoparticles in mammalian germline stem cells*. Toxicological Sciences **88**(2): 412-419.
35. Broughton, W. J., G. Hernández, M. Blair, S. Beebe, P. Gepts and J. Vanderleyden, 2003 - *Beans (Phaseolus spp.) – model food legumes*. Plant and Soil **252**(1): 55-128.
36. Byrd, G. T. and P. A. May, 2000 - *Physiological Comparisons of Switchgrass Cultivars Differing in Transpiration Efficiency*. Crop Sci. **40**(5): 1271-1277.
37. Carović-Stanko, K., S. Orlić, O. Politeo, F. Strikić, I. Kolak, M. Milos and Z. Satovic, 2010 - *Composition and antibacterial activities of essential oils of seven Ocimum taxa*. Food Chemistry **119**(1): 196-201.

BCU CENTRAL UNIVERSITY LIBRARY

38. Castellano, J. J., S. M. Shafii, F. Ko, G. Donate, T. E. Wright, R. J. Mannari, W. G. Payne, D. J. Smith and M. C. Robson, 2007 - *Comparative evaluation of silver-containing antimicrobial dressings and drugs*. Int Wound J 4(2): 114-122.

39. Celie, P. H. N., S. E. van Rossum-Fikkert, W. J. van Dijk, K. Brejc, A. B. Smit and T. K. Sixma, 2004 - *Nicotine and Carbamylcholine Binding to Nicotinic Acetylcholine Receptors as Studied in AChBP Crystal Structures*. Neuron **41**(6): 907-914.

40. Chaiharn, M. and S. Lumyong, 2011 - *Screening and Optimization of Indole-3-Acetic Acid Production and Phosphate Solubilization from Rhizobacteria Aimed at Improving Plant Growth.* Current Microbiology **62**(1): 173-181.

41. Chanda, S., Baravalia. Y., M. Kaneria and K. Rakholiya, 2010 - *Fruit and vegetable peels – strong natural source of antimicrobics*. Current research, technology and education topics in apllied microbiology and microbial biotechnology. M.-V. A. Badajoz, Formatex, Spain.

42. Chaurasia, S. and R. Saxena, 2012 - *Antibacterial Activity of Four Different Varieties of Green Beans*. Res J Pharm Biol Che Sci **3**: 70-74.

43. Chen, Y. P., P. D. Rekha, A. B. Arun, F. T. Shen, W. A. Lai and C. C. Young, 2006 - *Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities*. Appl. Soil Ecol. **34**(1): 33-41.

44. Chen, Y. P., P. D. Rekha, A. B. Arun, F. T. Shen, W. A. Lai and C. C. Young, 2006 - *Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities.* Applied Soil Ecology **34**(1): 33-41.

BCU MSU CENTRAL UNIVERSITY LIBRARY

45. Cho, K.-H., J.-E. Park, T. Osaka and S.-G. Park, 2005 - *The study of antimicrobial activity and preservative effects of nanosilver ingredient.* Electrochimica Acta **51**(5): 956-960.
46. Chopra, I., 2000 - *New drugs for superbugs*. Microbiology Today **47**: 4–6.
47. Cocking, E. C., 2003 - *Endophytic colonization of plant roots by nitrogen-fixing bacteria*. Plant Soil **252**(1): 169-175.
48. Cocking, E. C., 2003 - *Endophytic colonization of plant roots by nitrogen-fixing bacteria*. Plant and Soil **252**(1): 169-175.
49. Compant, S., D. Brion, N. Jerzy, C. Christophe and A. B. Essaïd, 2005 - *Use of Plant Growth-Promoting Bacteria for biocontrol of plant diseases: principles, mechanisms of action and future prospects.* Appl. Environ. Microbiol. **71**(9): 4951-4959.
50. Compant, S., C. Clément and A. Sessitsch, 2010 - *Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization*. Soil Biology and Biochemistry **42**(5): 669-678.
51. Cowan, M. M., 1999 - *Plant Products as Antimicrobial Agents*. Clinical Microbiology Reviews **12**(4): 564-582.
52. Dahiya, S. S., S. S. Sheoran and S. K. Sharma, 2010 - *Antibacterial activity of Amaranthus hybridus linn. root extracts*. IJABPT **1**: 46–49.
53. Dambolena, J. S., M. P. Zunino, A. G. López, H. R. Rubinstein, J. A. Zygadlo, J. W. Mwangi, G. N. Thoithi, I. O. Kibwage, J. M. Mwalukumbi and S. T. Kariuki, 2010 - *Essential oils composition of Ocimum basilicum L. and Ocimum gratissimum L. from Kenya and their inhibitory effects on growth and fumonisin production by*

BCU JOSEPH CENTRAL UNIVERSITY LIBRARY

Fusarium verticillioides. Innovative Food Science & Emerging Technologies **11**(2): 410-414.

54. Daniel, M.-C. and D. Astruc, 2003 - *Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology*. Chemical Reviews **104**(1): 293-346.
55. Dashti, N., F. Zhang, R. Hynes and D. L. Smith, 1997 - *Application of plant growth-promoting rhizobacteria to soybean (Glycine max [L] Merr) increases protein and dry matter yield under short-season conditions*. Plant and Soil **188**(1): 33-41.
56. Davidson, A. L., E. Dassa, C. Orelle and J. Chen, 2008 - *Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems*. Microbiology and Molecular Biology Reviews **72**(2): 317-364.
57. De Freitas, J. R., M. R. Banerjee and J. J. Germida, 1997 - *Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (Brassica napus L.)*. Biol. Fertil. Soils **24**: 358-364.
58. Deka Boruah, H. P., B. K. Rabha, N. Saikia and B. S. Dileep Kumar, 2003 - *Fluorescent Pseudomonas influences palisade mesophyll development and spatial root development in Phaseolus vulgaris*. Plant Soil **256**(2): 291-301.
59. Dey, K. B., 1988 - *Phosphate solubilizing organisms in improving fertility status*. Biofertilizers: Potentialities and Problems. S. P. Sen and P. Palit. Calcutta, Plant Physiology Forum, Naya Prokash: 237–248.
60. Dey, R., K. K. Pal, D. M. Bhatt and S. M. Chauhan, 2004 - *Growth promotion and yield enhancement of peanut (Arachis hypogaea L.) by*

BCU IASI / CENTRAL UNIVERSITY LIBRARY

application of plant growth-promoting rhizobacteria. Microbiological Research **159**(4): 371-394.

61. Dorman, H. J. D. and S. G. Deans, 2000 - *Antimicrobial agents from plants: antibacterial activity of plant volatile oils*. Journal of Applied Microbiology **88**(2): 308-316.
62. Doss, A. and M. Pugualenthi, 2012 - *Evaluation of antioxydant activity and phytochemical screening of Malus domestica Borkh (apple) and Phaseolus vulgaris L. (green beans)*. Journal of Pharmaceutical and Scientific Innovation **3**: 1-4.
63. Egorova, E. M., A. A. Revina, T. N. Rostovshchikova and O. I. Kiseleva, 2001 - *Bactericidal and Catalytic Properties of Stable Metal Nanoparticles in Reverse Micelles*. estn. Mosk. Univ., Ser. 2: Khim. **42**(5): 332–338.
64. Elsome, A. M., J. M. T. Hamilton-Miller, W. Brumfitt and W. C. Noble, 1996 - *Antimicrobial activities in vitro and in vivo of transition element complexes containing gold (I) and osmium (VI)*. Journal of Antimicrobial Chemotherapy **37**(5): 911-918.
65. Esitken, A., L. Pirlak, M. Turan and F. Sahin, 2006 - *Effects of floral and foliar application of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrition of sweet cherry*. Sci. Hortic. **110**(4): 324-327.
66. Esumi, K., N. Takei and T. Yoshimura, 2003 - *Antioxidant-potentiality of gold–chitosan nanocomposites*. Colloids and Surfaces B: Biointerfaces **32**(2): 117-123.
67. Fernández, L., P. Zalba, M. Gómez and M. Sagardoy, 2007 - *Phosphate-solubilization activity of bacterial strains in soil and their*

BCU IASI / CENTRAL UNIVERSITY LIBRARY

effect on soybean growth under greenhouse conditions. Biology and Fertility of Soils **43**(6): 805-809.

68. Fu, G., P. S. Vary and C.-T. Lin, 2005 - *Anatase TiO2 Nanocomposites for Antimicrobial Coatings*. The Journal of Physical Chemistry B **109**(18): 8889-8898.
69. Gamalero, E., G. Lingua, G. Berta and P. Lemanceau, 2009 - *Methods for Studying Root Colonization by Introduced Beneficial Bacteria Sustainable Agriculture*. E. Lichtfouse, M. Navarrete, P. Debaeke, S. Véronique and C. Alberola, Springer Netherlands: 601-615.
70. Gargova, S., Z. Roshkova and G. Vancheva, 1997 - *Screening of fungi for phytase production*. Biotechnology Techniques **11**(4): 221-224.
71. Glick, B. R., 1995 - *The enhancement of plant growth by free living bacteria*. Can J Microbiol.(41): 109-117.
72. Glick, B. R., 1999 - *Biochemical and genetic mechanisms used by plant growth-promoting bacteria*. River Edge, NJ, ICP.
73. Glick, B. R., D. M. Penrose and J. Li, 1998 - *A Model For the Lowering of Plant Ethylene Concentrations by Plant Growth-promoting Bacteria*. Journal of Theoretical Biology **190**(1): 63-68.
74. Goldstein, A. H., 1986 - *Bacterial solubilization of mineral phosphates: historical perspective and future prospects*. Am J Altern Agri **1**: 51–57.
75. Gong, P., H. Li, X. He, K. Wang, J. Hu, W. Tan, S. Zhang and X. Yang, 2007 - *Preparation and antibacterial activity of Fe3O4 Ag nanoparticles*. Nanotechnology **18**(28): 285604.

BCU MST CENTRAL UNIVERSITY LIBRARY

76. Goodman, C. M., C. D. McCusker, T. Yilmaz and V. M. Rotello, 2004 - *Toxicity of Gold Nanoparticles Functionalized with Cationic and Anionic Side Chains*. Bioconjugate Chemistry **15**(4): 897-900.

77. Gordon, S. A. and R. P. Weber, 1951 - *Colorimetric Estimation of Indoleacetic Acid*. Plant Physiol. **26**(1): 192-195.

78. Greaves, M. P. and D. M. Webley, 1965 - *A Study of the Breakdown of Organic Phosphates by Micro-organisms from the Root Region of Certain Pasture Grasses*. Journal of Applied Microbiology **28**(3): 454-465.

79. Gupta, R., R. Singal, A. Sankar, R. M. Chander and R. S. Kumar, 1994 - *A modified plate assay for screening phosphate solubilizing microorganisms*. J. Gen. Appl. Microbiol. **40**: 255–260.

80. Gutiérrez-Mañero, F. J., B. Ramos-Solano, A. Probanza, J. R. Mehouachi, F. Tadeo and M. Talon, 2001 - *The plant-growth-promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins*. Physiologia Plantarum **111**(2): 206-211.

81. Haas, D. and C. Keel, 2003 - *Regulation of antibiotic production in root-colonizing Pseudomonas spp. and relevance for biological control of plant disease*. Annual Review of Phytopathology **41**(1): 117-153.

82. Hahn, G., B. Kaup, S. Bringer-Meyer and H. Sahm, 2003 - *A zinc-containing mannitol-2-dehydrogenase from Leuconostoc pseudomesenteroides ATCC 12291: purification of the enzyme and cloning of the gene*. Archives of Microbiology **179**(2): 101-107.

BCU MAIN CENTRAL UNIVERSITY LIBRARY

83. Hammer, K. A., C. F. Carson and T. V. Riley, 1999 - *Antimicrobial activity of essential oils and other plant extracts*. J. Appl. Microbiol. **86**(6): 985-990.

84. Han, H. S. and K. D. Lee, 2005 - *Plant Growth Promoting Rhizobacteria Effect on Antioxidant Status, Photosynthesis, Mineral Uptake and Growth of Lettuce under Soil Salinity*. Res. J. Agric. & Biol. Sci. **1**(3): 210-215.

85. Hansen, A. P., 1994 - *Symbiotic N2 Fixation of Crop Legumes*. Weikershein, Germany, Margraf Verlag.

86. Heidari, M. and A. Golpayegani, 2012 - *Effects of water stress and inoculation with plant growth promoting rhizobacteria (PGPR) on antioxidant status and photosynthetic pigments in basil (Ocimum basilicum L.)*. Journal of the Saudi Society of Agricultural Sciences **11**(1): 57-61.

87. Helander, I. M., E. L. Nurmiaho-Lassila, R. Ahvenainen, J. Rhoades and S. Roller, 2001 - *Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria*. International Journal of Food Microbiology **71**(2–3): 235-244.

88. Hernández-Sierra, J. F., F. Ruiz, D. C. Cruz Pena, F. Martínez-Gutiérrez, A. E. Martínez, A. de Jesús Pozos Guillén, H. Tapia-Pérez and G. Martínez Castañón, 2008 - *The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold*. Nanomedicine: Nanotechnology, Biology and Medicine **4**(3): 237-240.

89. Howie, W. J., R. J. Cook and D. M. Weller, 1987 - *Effects of soil matric potential and cell motility on wheat root colonization by*

BCU MSU CENTRAL UNIVERSITY LIBRARY

fluorescent pseudomonads suppressive to take-all. Phytopathology 77(2): 286-292.

90. Huie, C., 2002 - *A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants.* Analytical and Bioanalytical Chemistry **373**(1-2): 23-30.
91. Hussain, A. J., F. Anwar, S. T. H. Sheraz and R. Przybylski, 2008 - *Chemical composition, antioxidant and antimicrobial activities of basil (Ocimum basilicum) essential oils depends on seasonal variations.* Food Chemistry **108** 986-995.
92. Hwangbo, H., R. D. Park, Y. W. Kim, Y. S. Rim, K. H. Park, T. H. Kim, J. S. Suh and K. Y. Kim, 2003 - *2-Ketogluconic Acid Production and Phosphate Solubilization by Enterobacter intermedium.* Current Microbiology **47**(2): 0087-0092.
93. Hwangbo, H., R. D. Park, Y. W. Kim, Y. S. Rim, K. H. Park, T. H. Kim, J. S. Suh and K. Y. Kim, 2003 - *2-Ketogluconic Acid Production and Phosphate Solubilization by Enterobacter intermedium.* Current Microbiology **47**(2): 87-92.
94. Ianos, R., D. Peptenatu and D. Zamfir, 2009 - *Respect for environment and sustainable development.* Carpathian Journal of Earth and Environmental Sciences **4**(1): 81 - 93.
95. Ibrahim, T. A., L. Ajala, F. O. Adetuyi and B. Jude-Ojei, 2009 - *Assessment of the antibacterial activity of Vernonia amygdalina and Occimum gratissimum leaves on selected food borne pathogens.* Elec J Env Agricult Food Chem **8**(11): 1212–1218.
96. Igloi, G. L. and R. Brandsch, 2003 - *Sequence of the 165-kilobase catabolic plasmid pAO1 from Arthrobacter nicotinovorans and*

BCU UNIVERSITY CENTRAL LIBRARY

identification of a pAO1-dependent nicotine uptake system. J Bacteriol(185): 1976-1986.

97. Illmer, P., A. Barbato and F. Schinner, 1995 - *Solubilization of hardly-soluble AlPO4 with P-solubilizing microorganisms.* Soil Biol Biochem **27**: 265–270.
98. Jain, R. A. S., S. S. P. I. Gupta and R. Gabrani, 2011 - *Solanum nigrum: current perspectives on therapeutic properties.* Altern Med Rev **16**: 78–85.
99. Jones, D. L., 1998 - *Organic acids in the rhizosphere – a critical review.* Plant and Soil **205**(1): 25-44.
100. Kaiser, W. M., 1979 - *Reversible inhibition of the Calvin cycle and activation of the oxidative pentose phosphate cycle in isolated chloroplast by hydrogen peroxide.* Planta **145**: 377-382.
101. Karaman, İ., F. Şahin, M. Güllüce, H. Öğütçü, M. Şengül and A. Adıgüzel, 2003 - *Antimicrobial activity of aqueous and methanol extracts of Juniperus oxycedrus L.* Journal of Ethnopharmacology **85**(2–3): 231-235.
102. Katyal, J. and B. Sharma, 1980 - *A new technique of plant analysis to resolve iron chlorosis.* Plant Soil **55**(1): 105-119.
103. Katznelson, H., E. A. Peterson and J. W. Rovatt, 1962 - *Phosphate dissolving microoganisms on seed and in the root zone of plants.* Can J Bot **40**: 1181–1186.
104. Kavino, M., S. Harish, N. Kumar, D. Saravanakumar and R. Samiyappan, 2010 - *Effect of chitinolytic PGPR on growth, yield and physiological attributes of banana (Musa spp.) under field conditions.* Appl. Soil Ecol. **45**(2): 71-77.

BCU IASRI / CENTRAL UNIVERSITY LIBRARY

105. Kean, T. and M. Thanou, 2010 - *Biodegradation, biodistribution and toxicity of chitosan.* Advanced Drug Delivery Reviews **62**(1): 3-11.

106. Khan, M. M. K., M. Bhuiyan, S. M. Kabir, Y. Oki and T. Adachi, 2006 - *Effect of selected treatments on the production of rice (Oryza sativa) in acid sulfate soils in a stimulation study.* Jpn J Trop Agric **50**: 109–115.

107. Khan, M. S., A. Zaidi and J. Mussarat, 2009 - *Microbial Strategies for Crop Improvement*, Springer.

108. Kim, J., M. R. Marshall and C. Wei, 1995 - *Antibacterial activity of some essential oil components against five foodborne pathogens.* Journal of Agricultural and Food Chemistry **43**(11): 2839-2845.

109. Kim, J. S., E. Kuk, K. N. Yu, J.-H. Kim, S. J. Park, H. J. Lee, S. H. Kim, Y. K. Park, Y. H. Park, C.-Y. Hwang, Y.-K. Kim, Y.-S. Lee, D. H. Jeong and M.-H. Cho, 2007 - *Antimicrobial effects of silver nanoparticles.* Nanomedicine: Nanotechnology, Biology and Medicine **3**(1): 95-101.

110. Kloepper, J. W., A. Gutiérrez-Estrada and J. A. McInroy, 2007 - *Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria.* Canadian Journal of Microbiology **53**(2): 159-167.

111. Kloepper, J. W., R. Lifshitz and R. M. Zablotowicz, 1989 - *Free-living bacterial inocula for enhancing crop productivity.* Trends Biotechnol.(7): 39-43.

112. Kloepper, J. W., R. M. Zablotowicz, E. M. Tipping and R. Lifshitz, 1991 - *Plant growth promotion mediated by bacterial rhizosphere colonizers.* The rhizosphere and plant growth. D. L. Keister and P. B.

BCU/CENTRAL UNIVERSITY LIBRARY

Cregan. Dordrecht, The Netherlands, Kluwer Academic Publishers: 315–326.

113. Knobloch, K., A. Pauli, B. Iberl, H. Weigand and N. Weis, 1989 - *Antibacterial and Antifungal Properties of Essential Oil Components.* Journal of Essential Oil Research **1**(3): 119-128.

114. Kobayashi, D. Y. and J. D. Palumbo, 2000 - *Bacterial endophytes and their effects on plants and uses in agriculture.* Microbial endophytes. C. W. James and J. F. J. White. New York, Marcel Dekker Inc.

115. Kodama, Y., H. Yamamoto, N. Amano and T. Amachi, 1992 - *Reclassification of Two Strains of Arthrobacter oxydans and Proposal of Arthrobacter nicotinovorans sp. nov.* International Journal of Systematic Bacteriology **42**(2): 234-239.

116. Koffi-Nevrya, R. K., K. C. Nangabc, Z. Y. Koussémona and G. Y. Loukoubc, 2012 - *Antibacterial activity of two bell pepper extracts: Capsicum annuum L and Capsicum frutescens.* Int J Food Prop **15**: 961–971.

117. Kong, M., X. G. Chen, K. Xing and H. J. Park, 2010 - *Antimicrobial properties of chitosan and mode of action: A state of the art review.* International Journal of Food Microbiology **144**(1): 51-63.

118. Kothari, S. K., A. K. Bhattacharya and S. Ramesh, 2004 - *Essential oil yield and quality of methyl eugenol rich Ocimum tenuiflorum L.f. (syn. O. sanctum L.) grown in south India as influenced by method of harvest.* Journal of Chromatography A **1054**(1–2): 67-72.

119. Kühn, A., S. Yu and F. Giffhorn, 2006 - *Catabolism of 1,5-Anhydro-d-Fructose in Sinorhizobium morelense S-30.7.5: Discovery, Characterization, and Overexpression of a New 1,5-Anhydro-d-*

BCU MAIN CENTRAL UNIVERSITY LIBRARY

Fructose Reductase and Its Application in Sugar Analysis and Rare Sugar Synthesis. Applied and Environmental Microbiology **72**(2): 1248-1257.

120. Kuklinsky-Sobral, J., W. L. Araújo, R. Mendes, I. O. Geraldi, A. A. Pizzirani-Kleiner and J. L. Azevedo, 2004 - *Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion*. Environ. Microbiol. **6**(12): 1244-1251.

121. Kumar, S., G. D. Bagchi and M. P. Darokar, 1997 - *Antibacterial Activity Observed in the Seeds of Some Coprophilous Plants*. Pharmaceutical Biology **35**(3): 179-184.

122. Kurihara, S., K. Kato, K. Asada, H. Kumagai and H. Suzuki, 2010 - *A Putrescine-Inducible Pathway Comprising PuuE-YneI in Which γ-Aminobutyrate Is Degraded into Succinate in Escherichia coli K-12*. Journal of Bacteriology **192**(18): 4582-4591.

123. Kvitek, L., A. Panaček, J. Soukupova, M. Kolar, R. Večerova, R. Prucek, M. Holecova and R. Zboril, 2008 - *Effect of Surfactants and Polymers on Stability and Antibacterial Activity of Silver Nanoparticles (NPs)*. The Journal of Physical Chemistry C **112**(15): 5825-5834.

124. Lahariya, A. K. and J. T. Rao, 1979 - *In Vitro antimicrobial studies of the essential oils of Cyperus scariosus and Ocimum basilicum*. Indian Drugs **16**: 150-152.

125. Laheurte, F. and J. Berthelin, 1988 - *Effect of a phosphate-solubilizing bacteria on maize growth and root exudation over four levels of labile phosphorus*. Plant and Soil **105**: 11–17.

BCU IASI CENTRAL UNIVERSITY LIBRARY

126. Lambrecht, M., Y. Okon, A. Vande Broek and J. Vanderleyden, 2000 - *Indole-3-acetic acid: a reciprocal signalling molecule in bacteria–plant interactions*. Trends in Microbiology **8**(7): 298-300.

127. Lee, D., R. E. Cohen and M. F. Rubner, 2005 - *Antibacterial Properties of Ag Nanoparticle Loaded Multilayers and Formation of Magnetically Directed Antibacterial Microparticles*. Langmuir **21**(21): 9651-9659.

128. Lee, H. Y., H. K. Park, Y. M. Lee, K. Kim and S. B. Park, 2007 - *A practical procedure for producing silver nanocoated fabric and its antibacterial evaluation for biomedical applications*. Chemical Communications **28**: 2959-2961.

129. Lee, K. D., E. J. Gray, F. Mabood, W. J. Jung, T. Charles and S. R. Clark, 2008 - *The class IId bacteriocin thuricin-17 increases plant growth*. Planta: DOI 10.1007/s00425-00008-00870-00426.

130. Liu, Z. L. and J. B. Sinclair, 1993 - *Colonization of soybean roots by Bacillus megaterium B153–2-2*. Soil Biol. Biochem. **25**: 849–855.

131. Lok, C. N., C. M. Ho, R. Chen, Q. Y. He, W. Y. Yu, H. Z. Sun, P. K. H. Tam, J. F. Chiu and C. M. Che, 2006 - *Proteomic analysis of the mode of antibacterial action of silver nanoparticles*. Journal of Proteome Research **5**(4): 916-924.

132. Lomovskaya, O. and K. A. Bostian, 2006 - *Practical applications and feasibility of efflux pump inhibitors in the clinic—A vision for applied use*. Biochemical Pharmacology **71**(7): 910-918.

133. Lorenzi, V., A. Muselli, A. F. Bernardini, L. Berti, J.-M. Pagès, L. Amaral and J.-M. Bolla, 2009 - *Geraniol Restores Antibiotic Activities*

BCU JASH CENTRAL UNIVERSITY LIBRARY

against Multidrug-Resistant Isolates from Gram-Negative Species. Antimicrobial Agents and Chemotherapy **53**(5): 2209-2211.

134. Lynch, J. M., 1990 - *The rhizosphere*. New York, John Wiley.

135. Maiyo, Z. C., R. M. Ngure, J. C. Matasyoh and R. Chepkorir, 2010 - *Phytochemical constituents and antimicrobial activity of leaf extracts of three Amaranthus plant species*. Afr J Biotechnol **9**: 3178–3182.

136. Majeti N.V, R. K., 2000 - *A review of chitin and chitosan applications*. Reactive and Functional Polymers **46**(1): 1-27.

137. Maksimov, I., R. Abizgildina and L. Pusenkova, 2011 - *Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens (review)*. Applied Biochemistry and Microbiology **47**(4): 333-345.

138. Malphettes, L., C. C. Weber, M. D. El-Baba, R. G. Schoenmakers, D. Aubel, W. Weber and M. Fussenegger, 2005 - *A novel mammalian expression system derived from components coordinating nicotine degradation in arthrobacter nicotinovorans pAO1*. Nucleic Acids Research **33**(12): e107.

139. Mandal, T. K., M. S. Fleming and D. R. Walt, 2002 - *Preparation of Polymer Coated Gold Nanoparticles by Surface-Confined Living Radical Polymerization at Ambient Temperature*. Nano Letters **2**(1): 3-7.

140. Mann, C. M. and J. L. Markham, 1998 - *A new method for determining the minimum inhibitory concentration of essential oils*. Journal of Applied Microbiology **84**(4): 538-544.

141. Marambio-Jones, C. and E. M. V. Hoek, 2010 - *A review of the antibacterial effects of silver nanomaterials and potential implications*

BCU IASI / CENTRAL UNIVERSITY LIBRARY
B.C.U. M. EMINESCU IAȘI

for human health and the environment. Journal of Nanoparticle Research **12**(5): 1531-1551.

142. Marchal, S. and G. Branlant, 1999 - *Evidence for the Chemical Activation of Essential Cys-302 upon Cofactor Binding to Nonphosphorylating Glyceraldehyde 3-Phosphate Dehydrogenase from Streptococcus mutans.* Biochemistry **38**(39): 12950-12958.

143. Marini, M., S. De Niederhausern, R. Iseppi, M. Bondi, C. Sabia, M. Toselli and F. Pilati, 2007 - *Antibacterial activity of plastics coated with silver-doped organic-inorganic hybrid coatings prepared by sol-gel processes.* Biomacromolecules **8**(4): 1246-1254.

144. Marotti, M., R. Piccaglia and E. Giovanelli, 1996 - *Differences in Essential Oil Composition of Basil (Ocimum basilicum L.) Italian Cultivars Related to Morphological Characteristics.* Journal of Agricultural and Food Chemistry **44**(12): 3926-3929.

145. Martínez-Castañón, G., N. Niño-Martínez, F. Martínez-Gutierrez, J. Martínez-Mendoza and F. Ruiz, 2008 - *Synthesis and antibacterial activity of silver nanoparticles with different sizes.* Journal of Nanoparticle Research **10**(8): 1343-1348.

146. Matasyoh, J. C., Z. C. Maiyo, R. M. Ngure and R. Chepkorir, 2009 - *Chemical composition and antimicrobial activity of the essential oil of Coriandrum sativum.* Food Chemistry **113**(2): 526-529.

147. Matsumura, Y., K. Yoshikata, S. Kunisaki and T. Tsuchido, 2003 - *Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate.* Applied and Environmental Microbiology **69**(7): 4278-4281.

BCU JASTI CENTRAL UNIVERSITY LIBRARY

148. Mbaveng, A. T., B. Ngameni, V. Kuete, I. K. Simo, P. Ambassa, R. Roy, M. Bezabih, F.-X. Etoa, B. T. Ngadjui, B. M. Abegaz, J. J. M. Meyer, N. Lall and V. P. Beng, 2008 - *Antimicrobial activity of the crude extracts and five flavonoids from the twigs of Dorstenia barteri (Moraceae)*. Journal of Ethnopharmacology **116**(3): 483-489.

149. McCully, M. E., 2001 - *Niches for bacterial endophytes in crop plants: a plant biologist's view*. Functional Plant Biology **28**(9): 983-990.

150. Meijnen, J.-P., J. H. de Winde and H. J. Ruijssenaars, 2009 - *Establishment of Oxidative d-Xylose Metabolism in Pseudomonas putida S12*. Applied and Environmental Microbiology **75**(9): 2784-2791.

151. Mihalache, G., N. Munteanu, S. Dunca, M. Mihasan and M. Stefan, 2012 - *Phosphate-solubilising rhizobacteria associated with Phaseolus coccineus L. rhizosphere*. Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară **TOM XIII**(Fasc. 3): 65-70.

152. Morones, J. R., J. L. Elechiguerra, A. Camacho, K. Holt, J. B. Kouri, J. T. Ramirez and M. J. Yacaman, 2005 - *The bactericidal effect of silver nanoparticles*. Nanotechnology **16**(10): 2346-2353.

153. Mukerji, K. G., C. Manoharachary and J. Singh, 2006 - *Microbial Activity in the Rhizosphere (Soil Biology)*, Springer.

154. Newman, D. J. and G. M. Cragg, 2007 - *Natural Products as Sources of New Drugs over the Last 25 Years*⊥ Journal of Natural Products **70**(3): 461-477.

155. Ngassoum, M. B., J. J. Essia-Ngang, L. N. Tatsadjieu, L. Jirovetz, G. Buchbauer and O. Adjoudji, 2003 - *Antimicrobial study of essential*

BCU IAŞI CENTRAL UNIVERSITY LIBRARY

oils of Ocimum gratissimum leaves and Zanthoxylum xanthoxyloides fruits from Cameroon. Fitoterapia **74**(3): 284-287.

156. Nkosi, C. Z., A. R. Opoku and S. E. Terblanche, 2005 - *Effect of pumpkin seed (Cucurbita pepo) protein isolate on the activity levels of certain plasma enzymes in CCl4-induced liver injury in low-protein fed rats*. Phytotherapy Research **19**(4): 341-345.

157. Ongena, Daayf, Jacques, Thonart, Benhamou, Paulitz, Cornélis, Koedam and Bélanger, 1999 - *Protection of cucumber against Pythium root rot by fluorescent pseudomonads: predominant role of induced resistance over siderophores and antibiosis*. Plant Pathology **48**(1): 66-76.

158. Ongena, M., E. Jourdan, A. Adam, M. Paquot, A. Brans, B. Joris, J.-L. Arpigny and P. Thonart, 2007 - *Surfactin and fengycin lipopeptides of Bacillus subtilis as elicitors of induced systemic resistance in plants*. Environmental Microbiology **9**(4): 1084-1090.

159. Ono, T., M. Kashimura, K. Suzuki, R. Oyauchi, J. Miyachi, H. Ikuta, H. Kawauchi, T. Akashi, T. Asaka and S. Morimoto, 2004 - *In vitro and in vivo antibacterial activities of the tricyclic ketolide te-802 and its analogues*. I. J Antibiot (Tokyo) **57**(8): 518–527.

160. Ordoñez, A. A. L., J. D. Gomez, N. M. Cudmani, M. A. Vattuone and M. I. Isla, 2003 - *Antimicrobial Activity of Nine Extracts of Sechium edule (Jacq.) Swartz*. Microb Ecol Health Dis **15**: 33–39.

161. Orhan, E., A. Esitken, S. Ercisli, M. Turan and F. Sahin, 2006 - *Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry*. Sci. Hortic. **111**(1): 38-43.

BCU UNIVERSITY CENTRAL LIBRARY

162. Oswald, A., P. Calvo Velez, D. Zúñiga Dávila and J. Arcos Pineda, 2010 - *Evaluating soil rhizobacteria for their ability to enhance plant growth and tuber yield in potato*. Ann. Appl. Biol. **157**(2): 259-271.

163. Pages, J.-M., J.-P. Lavigne, V. Leflon-Guibout, E. Marcon, F. Bert, L. Noussair and M.-H. Nicolas-Chanoine, 2009 - *Efflux Pump, the Masked Side of ß-Lactam Resistance in Klebsiella pneumoniae Clinical Isolates*. PLoS ONE **4**(3): e4817.

164. Pal, S., Y. K. Tak and J. M. Song, 2007 - *Does the Antibacterial Activity of Silver Nanoparticles Depend on the Shape of the Nanoparticle? A Study of the Gram-Negative Bacterium Escherichia coli*. Appl. Environ. Microbiol. **73**(6): 1712-1720.

165. Panácek, A., M. Kolár, R. Vecerová, R. Prucek, J. Soukupová, V. Krystof, P. Hamal, R. Zboril and L. Kvítek, 2009 - *Antifungal activity of silver nanoparticles against Candida spp*. Biomaterials **30**(31): 6333-6340.

166. Pantujit, S. and N. Pongsilp, 2010 - *Phosphatase activity and affects of phosphate-solubilizing bacteria on yield and uptake of phosphorus in corn*. World Applied Sciences Journal **8**(2): 429-435.

167. Papadopoulos, C. J., C. F. Carson, B. J. Chang and T. V. Riley, 2008 - *Role of the MexAB-OprM Efflux Pump of Pseudomonas aeruginosa in Tolerance to Tea Tree (Melaleuca alternifolia) Oil and Its Monoterpene Components Terpinen-4-ol, 1,8-Cineole, and α-Terpineol*. Applied and Environmental Microbiology **74**(6): 1932-1935.

168. Park, J. H., N. Bolan, M. Megharaj and R. Naidu, 2011 - *Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil*. J. Hazard. Mater. **185**(2–3): 829-836.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

169. Patten, C. L. and B. R. Glick, 1996 - *Bacterial biosynthesis of indole-3-acetic acid.* Can. J. Microbiol. **42**(3): 207-220.

170. Pavithra, V. K. O. and L. Bhagya, 2009 - *Antibacterial activity of black pepper (Piper nigrum Linn.) with special reference to its mode of action bacteria.* Indian J Comp Microbiol Immunol Infect Dis **30**(1): 65–66.

171. Peng, S., C. Jatish, K. Biswas, P. Ladha and Y. Chen, 2002 - *Influence of rhizobial inoculation on photosynthesis and grain yield of rice.* Agron. J. **94**: 925–929.

172. Perez-Miranda, S., N. Cabirol, R. George-Tellez, L. S. Zamudio-Rivera and F. J. Fernandez, 2007 - *O-CAS, a fast and universal method for siderophore detection.* J Microbiol Methods **70**(1): 127-131.

173. Pérez, E., M. Sulbarán, M. M. Ball and L. A. Yarzábal, 2007 - *Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region.* Soil Biol. Biochem. **39**(11): 2905-2914.

174. Perni, S., C. Piccirillo, J. Pratten, P. Prokopovich, W. Chrzanowski, I. P. Parkin and M. Wilson, 2009 - *The antimicrobial properties of light-activated polymers containing methylene blue and gold nanoparticles.* Biomaterials **30**(1): 89-93.

175. Podile, A. R. and G. K. Kishore, 2006 - *Plant growth promoting rhizobacteria.* Plant-Associated Bacteria. S. S. Gnanamanickam. Dordrecht, The Netherlands, Springer: 195-231.

176. Ponmurugan, P. and C. Gopi, 2006 - *In vitro production of growth regulators and phosphatase activity by phosphate solubilizing bacteria.* Afr. J. Biotechnol. **5**(4): 348-350.

BCUAS CENTRAL UNIVERSITY LIBRARY

177. Prasad, G., A. Kuman, A. K. Singh, A. K. Bhattacharya, K. Singh and V. D. Sharma, 1986 - *Antimicrobial activity of essential oils of some Ocimum species and clove oil.* Fitoterapia(57): 429-432.

178. Probanza, A., J. A. Lucas, N. Acero and F. J. Gutierrez-Manero, 1996 - *The influence of native rhizobacteria on european alder (Alnus glutinosa [L.] Gaertn.) growth. I. Characterization of growth promoting and growth inhibiting bacterial strains.* Plant Soil(182): 59-66.

179. Queiroga, C. L., M. C. Teixeira Duarte, B. Baesa Ribeiro and P. M. de Magalhães, 2007 - *Linalool production from the leaves of Bursera aloexylon and its antimicrobial activity.* Fitoterapia **78**(4): 327-328.

180. Raghu, K. and I. C. MacRae, 1966 - *Occurrence of phosphate-dissolving microorganisms in the rhizosphere of rice plants and in submerged soils.* J Appl Bacteriol **29**: 582–586.

181. Rai, M., A. Yadav and A. Gade, 2009 - *Silver nanoparticles as a new generation of antimicrobials.* Biotechnology Advances **27**(1): 76-83.

182. Ramos, B., L. A. Lucas Garcia, A. Probanza, J. Domenech and F. J. Gutierrez Manero, 2003 - *Influence of an indigenous European alder (Alnus glutinosa (L.) Gaertn) rhizobacterium (Bacillus pumilus) on the growth of alder and its rhizosphere microbial community structure in two soils.* New Forests **25**: 149-159.

183. Rascio, N. and N. La Rocca, 2008 - *Biological Nitrogen Fixation.* Encyclopedia of Ecology. J. Editors-in-Chief: Sven Erik and F. Brian. Oxford, Academic Press: 412-419.

184. Rasouli Sadaghiani, M. H., M. Barin and F. Jalili, 2008 - *The Effect of PGPR Inoculation on the Growth of Wheat.* International Meeting on Soil Fertility Land Management and Agroclimatology, Turkey.

BCU AGRICULTURAL UNIVERSITY LIBRARY

185. Richardson, A. E., 1994 - *Soil microorganisms and phosphorus availability*. Soil Biota, Management in Sustainable Farming Systems. C. E. Pankhurst, B. M. Doube, V. V. S. R. Grupta and P. R. Grace. Melbourne, Australia, CSIRO: 50–62.

186. Richardson, A. E., 2001 - *Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants*. Australian Journal of Plant Physiology **28**(9): 897-906.

187. Rodríguez, H. and R. Fraga, 1999 - *Phosphate solubilizing bacteria and their role in plant growth promotion*. Biotechnol. Adv. **17**(4-5): 319-339.

188. Rodríguez, H. and R. Fraga, 1999 - *Phosphate solubilizing bacteria and their role in plant growth promotion*. Biotechnology Advances **17**(4-5): 319-339.

189. Rojas, A., G. Holguin, B. R. Glick and Y. Bashan, 2001 - *Synergism between Phyllobacterium sp. (N2-fixer) and Bacillus licheniformis (P-solubilizer), both from a semiarid mangrove rhizosphere*. FEMS Microbiology Ecology **35**(2): 181-187.

190. Roller, S. and N. Covill, 1999 - *The antifungal properties of chitosan in laboratory media and apple juice*. International Journal of Food Microbiology **47**(1–2): 67-77.

191. Rupp, M. E., T. Fitzgerald, N. Marion, V. Helget, S. Puumala, J. R. Anderson and P. D. Fey, 2004 - *Effect of silver-coated urinary catheters: efficacy, cost-effectiveness, and antimicrobial resistance*. American Journal of Infection Control **32**(8): 445-450.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

192. Saleh, S. S. and B. R. Glick, 2001 - *Involvement of gacS and ropS in the transcriptional regulation of the plant growth promoting bacteria Enterobacter cloacae CAL2 and UW4*. Can. J. Microbiol. **47**: 698-705.

193. Salisbury, F. B., 1994 - *The role of plant hormones*. Plant–Environment Interactions. R. E. Wilkinson. New York, USA, Marcel Dekker: 39–81.

194. Salvagiotti, F., K. G. Cassman, J. E. Specht, D. T. Walters, A. Weiss and A. Dobermann, 2008 - *Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review*. Field Crops Research **108**(1): 1-13.

195. Sannazzaro, A. I., O. A. Ruiz, E. O. Alberto and A. B. Menendez, 2006 - *Alleviation of salt stress in Lotus glaber by Glomus intraradices*. Plant and Soil **285**: 279-287.

196. Saravanakumar, D., M. Kavino, T. Raguchander, P. Subbian and R. Samiyappan, 2011 - *Plant growth promoting bacteria enhance water stress resistance in green gram plants*. Acta Physiologiae Plantarum **33**(1): 203-209.

197. Schie, C. N., M. Haring and R. Schuurink, 2007 - *Tomato linalool synthase is induced in trichomes by jasmonic acid*. Plant Molecular Biology **64**(3): 251-263.

198. Schwyn, B. and J. B. Neilands, 1987 - *Universal Chemical Assay for the Detection and Determination of Siderophores*. Anal. Biochem. **160**: 47-56.

199. Shariati, A., H. R. Pordeli, A. Khademian and M. Aydani, 2010 - *Evaluation of the antibacterial effects of Capsicum spp. extracts on the Multi-resistant Staphylococcus aureus strains*. J Plant Sci Res **17**: 10–16.

BCU JSC CENTRAL UNIVERSITY LIBRARY

200. Sharma, A. and B. N. Johri, 2003 - *Growth promoting influence of siderophore-producing Pseudomonas strains GRP3A and PRS9 in maize (Zea mays L.) under iron limiting conditions*. Microbiological Research **158**(3): 243-248.

201. Shrivastava, S., T. Bera, A. Roy, G. Singh, P. Ramachandrarao and D. Dash, 2007 - *Characterization of enhanced antibacterial effects of novel silver nanoparticles*. Nanotechnology **18**(22).

202. Singh, A. and P. Mishra, 1995 - *Microbial uptake of pentoses*. Microbial Pentose Utilization: Current Applications in Biotechnology. A. Singh, Mishra, P. Saint Louis, Elsevier: 99-145.

203. Singh, J. S., V. C. Pandey and D. P. Singh, 2011 - *Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development*. Agric. Ecosyst. Environ. **140**(3–4): 339-353.

204. Singh, S. and K. K. Kapoor, 1999 - *Inoculation with phosphate solubilizing microorganisms and a vesicular arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil*. Biol. Fertil. Soils **28**: 139–144.

205. Sixma, T. K. and A. B. Smit, 2003 - *Acetylcholine binding protein (AChBP): a secreted glial protein that provides a high-resolution model for the extracellular domain of pentameric ligand-gated ion channels*. Annual review of biophysics and biomolecular structure **32**: 311-334.

206. Smalla, K., A. Sessitsch and A. Hartmann, 2006 - *The Rhizosphere: soil compartment influenced by the root*. FEMS Microbiology Ecology **56**(2): 165-165.

BCU JASH CENTRAL UNIVERSITY LIBRARY

207. Sondi, I. and B. Salopek-Sondi, 2004 - *Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria.* J Colloid Interface Sci.(275): 177-182.

208. Sondi, I. and B. Salopek-Sondi, 2004 - *Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria.* Journal of Colloid and Interface Science **275**(1): 177-182.

209. Spaepen, S., J. Vanderleyden and R. Remans, 2007 - *Indole-3-acetic acid in microbial and microorganism-plant signaling.* FEMS Microbiology Reviews **31**(4): 425-448.

210. Stefan, M., S. Dunca, Z. Olteanu, L. Oprica, E. Ungureanu, L. Hritcu, M. Mihasan and D. Cojocaru, 2010 - *Soybean (Glycine max [L] Merr.) inoculation with Bacillus pumilus Rs3 promotes plant growth and increases seed protein yield: relevance for environmentally-friendly agricultural applications.* Carpathian Journal of Earth and Environmental Sciences **5**(1): 131 – 138.

211. Stefan, M., L. Hritcu, M. Mihasan, D. Pricop, I. Gostin, R. O. Olariu, D. Dunca and V. Melnig, 2011 - *Enhanced antibacterial effect of silver nanoparticles obtained by electrochemical synthesis in poly(amide-hydroxyurethane) media.* Journal of Materials Science: Materials in Medicine **22**(4): 789-796.

212. Stefan, M., M. Mihăşan and S. Dunca, 2008 - *Plant growth promoting rhizobacteria can inhibit the in vitro germination of Glycine max L. seeds.* Analele Ştiinţifice ale Universităţii Alexandru Ioan Cuza din Iaşi, Secţiunea II-a Genetică şi Biologie Moleculară **TOM IX**(Fasc. 3): 105-110.

BCU IAŞI / CENTRAL UNIVERSITY LIBRARY

213. Stefan, M., N. Munteanu, V. Stoleru and M. Mihasan, 2013 - *Effects of inoculation with plant growth promoting rhizobacteria on photosynthesis, antioxidant status and yield of runner bean.* Romanian Biotechnological Letters **18**(2): 8132-8143.

214. Stefan, M., N. Munteanu, V. Stoleru, M. Mihasan and L. Hritcu, 2013 - *Seed inoculation with plant growth promoting rhizobacteria enhances photosynthesis and yield of runner bean (Phaseolus coccineus L.).* Scientia Horticulturae **151**(0): 22-29.

215. Stefan, M., M. Zamfirache, C. Padurariu, E. Trută and I. Gostin, 2013 - *The composition and antibacterial activity of essential oils in three Ocimum species growing in Romania.* Central European Journal of Biology **8**(6): 600-608.

216. Stevenson, F. J. and M. A. Cole, 1999 - *Cycles of soil: Carbon, Nitrogen, Phosphorous, Sulfur, Micronutrients*. New York, Wiley.

217. Stoimenov, P. K., R. L. Klinger, G. L. Marchin and K. J. Klabunde, 2002 - *Metal Oxide Nanoparticles as Bactericidal Agents*. Langmuir **18**(17): 6679-6686.

218. Sullivan, R. and H. Zhao, 2007 - *Cloning, characterization, and mutational analysis of a highly active and stable l-arabinitol 4-dehydrogenase from Neurospora crassa.* Applied Microbiology and Biotechnology **77**(4): 845-852.

219. Suppakul, P., J. Miltz, K. Sonneveld and S. W. Bigger, 2003 - *Antimicrobial properties of basil and its possible application in food packing*. Journal of Agriculture and Food Chemistry **51**: 3197-3207.

BCU IAȘI / CENTRAL UNIVERSITY LIBRARY

220. Sylvia, D. M., J. J. Fuhrmann, P. G. Hartel and D. A. Zuberer, 1999 - *Principles and applications of soil microbiology*. New Jersey, Prentice Hall Inc., Upper Saddle River.

221. Thiel, J., L. Pakstis, S. Buzby, M. Raffi, C. Ni, D. J. Pochan and S. I. Shah, 2007 - *Antibacterial Properties of Silver-Doped Titania13*. Small **3**(5): 799-803.

222. Thomas, F. C., C. A. Woeng and B. J. J. Lugtenberg, 2008 - *Root Colonisation Following Seed Inoculation*. Plant Surface Microbiology. A. Varma, L. Abbott, D. Werner and R. Hampp. Berlin Heidelberg, Springer-Verlag: 13-35.

223. Toro, M., R. Azcón and R. Herrera, 1996 - *Effects on yield and nutrition of mycorrhizal and nodulated Pueraria phaseoloides exerted by P-solubilizing rhizobacteria*. Biol. Fertil. Soils **21**(1): 23-29.

224. Tortora, J., C. L. Case and B. R. Funke, 2002 - *Microbiology: An Introduction*. San Francisco, Benjamin Cummings.

225. Vessey, J. K., 2003 - *Plant growth promoting rhizobacteria as biofertilizers*. Plant Soil **255**(2): 571-586.

226. Vessey, J. K., 2003 - *Plant growth promoting rhizobacteria as biofertilizers*. Plant and Soil **255**(2): 571-586.

227. Voukeng, I., V. Kuete, J. Dzoyem, A. Fankam, J. A. Noumedem, J. Kuiate and J.-M. Pages, 2012 - *Antibacterial and antibiotic-potentiation activities of the methanol extract of some cameroonian spices against Gram-negative multi-drug resistant phenotypes*. BMC Research Notes **5**(1): 299.

BCU IASI CENTRAL UNIVERSITY LIBRARY

228. Wagner, H. and G. Ulrich-Merzenich, 2009 - *Synergy research: Approaching a new generation of phytopharmaceuticals*. Phytomedicine **16**(2–3): 97-110.

229. Walker, T. S., H. P. Bais, E. Grotewold and J. M. Vivanco, 2003 - *Root Exudation and Rhizosphere Biology*. Plant Physiology **132**(1): 44-51.

230. Wang, R. H., Z. G. Hu, Y. Liu, H. Lu, B. Fei, Y. S. Szeto, W. L. Chan, X. M. Tao and J. H. Xin, 2006 - *Self-Assembled Gold Nanoshells on Biodegradable Chitosan Fibers*. Biomacromolecules **7**(10): 2719-2721.

231. Weller, D. M. and L. S. Thomashow, 1994 - *Current Challenges in Introducing Beneficial Microorganisms into the Rhizosphere*. Molecular Ecology of Rhizosphere. E. O'Gara, D. N. Dowling and B. Boesten, VCH Verlagsgesellschaft mbH**:** 1-19.

232. Whipps, J. M., 2001 - *Microbial interactions and biocontrol in the rhizosphere*. Journal of Experimental Botany **52**(suppl 1): 487-511.

233. Williams, D., S. Ehrman and T. Pulliam Holoman, 2006 - *Evaluation of the microbial growth response to inorganic nanoparticles*. Journal of Nanobiotechnology **4**(1): 3.

234. Zahir, Z. A., M. Arshad and W. T. J. Frankenberger, 2003 - *Plant growth promoting rhizobacteria: applications and perspectives in agriculture*. Advances in Agronomy **81**: 97-168.

235. Zahir, Z. A., M. Arshad and W. T. J. Frankenberger, 2003 - *Plant growth promoting rhizobacteria: applications and perspectives in agriculture*. Adv. Agron. **81**: 97-168.

BCU ASIA CENTRAL UNIVERSITY LIBRARY

236. Zakaria, Z. A., H. M. Khairi, M. N. Somchit, M. R. Sulaiman, A. M. Mat Jais, I. Reezal, N. N. Mat Zaid, S. N. Z. Abdul Wahab, N. S. Fadzil, M. Abdullah and C. A. Fatimah, 2006 - *The in vitro antibacterial activity and brine shrimp toxicity of Manihot esculenta var. Sri Pontian extracts*. Int J Pharmacol **2**: 216–220.

237. Zamfirache, M. M., 2005 - *Fiziologie vegetala*. Iasi, Editura Azimuth.

238. Zhang, F., N. Dashti, R. K. Hynes and D. L. Smith, 1997 - *Plant growth-promoting rhizobacteria and soybean [Glycine max (L) Merr] growth and physiology at suboptimal root zone temperatures*. Ann. Bot. **79**(3): 243-249.

239. Zhang, X., M. Guo and H. Wu, 2009 - *Irradiation stability and cytotoxicity of gold nanoparticles for radiotherapy*. Journal: International Journal of Nanomedicine **4**(1): 165-173.

240. Zhang, Y., H. Peng, W. Huang, Y. Zhou and D. Yan, 2008 - *Facile preparation and characterization of highly antimicrobial colloid Ag or Au nanoparticles*. Journal of Colloid and Interface Science **325**(2): 371-376.

241. Zhao, G. J. and S. E. Stevens, 1998 - *Multiple parameters for the comprehensive evaluation of the susceptibility of Escherichia coli to the silver ion*. BioMetals **11**(1): 27-32.

242. Zheljazkov, V. D., C. L. Cantrell, B. Tekwani and S. I. Khan, 2007 - *Content, Composition, and Bioactivity of the Essential Oils of Three Basil Genotypes as a Function of Harvesting*. Journal of Agricultural and Food Chemistry **56**(2): 380-385.

BCU IASI / CENTRAL UNIVERSITY LIBRARY

APPLIED MICROBIOLOGY

FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS

Marius Ştefan

The research and educational book conceived as a monographic work "APPLIED MICROBIOLOGY: FROM PLANT GROWTH PROMOTION TO NEW NEUROPROTECTIVE DRUGS" is an example of applied microbiology/biotechnology, its main author and contributors presenting their own research experience. This book is structured in three chapters dedicated to plant-microbial interactions in the rhizosphere – strategies for plant growth promotion; antimicrobial applications of different natural and artificial compounds and new biotechnological applications of the metabolic derivatives from *Arthrobacter nicotinovorans*. The diversity of these apparently unrelated subjects, but having a common denominator, are reflecting the huge applicative aria of microbiology knowledge, of bacteria and other microorganisms with different metabolic particularities, used in different biotechnologies/nanobiotechnologies and their great potential to offer solutions at different problems in agriculture, medicine and even for its own branch – clinical microbiology.

Prof. dr. Veronica Lazar
Faculty of Biology
The University of Bucharest

www.editura.uaic.ro

9 786067 140910

Editura Universităţii „Alexandru Ioan Cuza" Iaşi

BCU IAŞI BIBLIOTECA CENTRALĂ UNIVERSITARĂ